Studies of plasticity after neuronal injury

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Studies of plasticity after neuronal injury

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Abstract

Neurons of the adult mammalian central nervous system (CNS) and peripheral nervous system (PNS) differ in their response to injury. PNS neurons have a robust growth response and are capable of regenerating their axons and reinnervating peripheral targets following nerve injury. However, despite this robust regenerative response, specificity of target reinnervation is often poor, leading to a suboptimal functional outcome. In contrast, injured CNS neurons have a poor growth response and completely fail to regenerate, but uninjured axons that survive the injury undergo some degree of spontaneous reorganisation that is accompanied by limited functional improvement. In theory, appropriate reorganisation of central connections could compensate for topographical errors in the periphery and enhancing the innate capacity for reorganisation after CNS injury could compensate for lost function. Thus, recovery from both types of injury may benefit from strategies to enhance CNS plasticity.

This Thesis makes use of an experimental strategy to promote plastic changes, that is, the enzyme chondroitinase ABC (ChABC) which degrades chondroitin sulphate proteoglycans – a major class of inhibitory extracellular matrix molecules. Viral vector technology was used to optimise the delivery of ChABC by gene transfer to host spinal cord cells. A lentiviral vector expressing the ChABC gene (LV-ChABC) was delivered to the adult rat spinal cord to achieve long-term delivery of active ChABC *in vivo*. After peripheral nerve injury, where two forelimb nerves were cut and repaired with different levels of inaccuracy, electrophysiological studies demonstrated that LV-ChABC injection led to significant plasticity of central connections, such as reorganisation of high and low threshold polysynaptic reflexes. This included preservation of the phenomenon of wind-up of flexor motor neurons, which was found to collapse after

nerve injury, as well as enhanced wind-up responses elicited by radial nerve stimulation. ChABC did not lead to any changes in reflex activity in uninjured animals. These results indicate that ChABC facilitates the amplification of compensatory changes in the spinal cord following injury to the periphery.

ChABC treatment was also investigated in the context of CNS injury. Following contusion injury, a clinically relevant model which mimics the functional and pathological characteristics typical of a human spinal cord injury, LV-ChABC injection led to significant functional improvements, including enhanced conduction, reorganisation of spinal reflexes below the lesion and improved performance on a sensorimotor behavioural task.

Finally, viral vector technology was used to develop a novel tool for the study of anatomical plasticity. By expressing the construct synaptopHluorin (SpH), lentiviral and adenoviral-associated viral vectors were used to label presynaptic terminals *in vitro* and *in vivo*. As the pHluorin component of the label is pH sensitive, this tool has the potential additional advantage of enabling the visualisation of synaptic function *in vivo*. The SpH vector was used to label a major motor pathway, the corticospinal tract, as well as sensory neurons, and to visualise their terminals as SpH-labelled puncta. Using this tool, it will be possible to study synaptogenesis in a quantifiable manner, with the potential to study the activity of these new synaptic terminals.

Thus, this Thesis uses a gene delivery approach to deliver ChABC to the spinal cord after central or peripheral neuronal injury, leading to the promotion of plasticity and functional repair. The development of a novel tool for visualising synapses is also described, presenting a novel opportunity for the study of plasticity after neuronal injury.

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AAV	adeno-associated virus
ANOVA	analysis of variance
BBB	Basso-Beattie-Bresnahan locomotor scale
BDA	biotinylated dextran amine
BDNF	brain-derived neurotrophic factor
C-4-S	chondroitin-4-sulphate
cAMP	cyclic adenosine monophosphate
CBP	complement binding protein
ChABC	chondroitinase ABC
CMV	cytomegalovirus
CNS	central nervous system
CPG	central pattern generator
cPPT	central polypurine tract
CRD	carbohydrate recognition domain
CS-GAG	chondroitin sulphate glycosaminoglycan
CSPG	chondroitin sulphate proteoglycan
CST	corticospinal tract
CTB	cholera toxin beta
DMEM	Dulbecco's modified Eagle's medium
DRG	dorsal root ganglion
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMG	electromyogram
ERK1	extracellular signal-related kinase 1
fMRI	functional magnetic resonance imaging
FN	fibronectin
GAG	glycosaminoglycan
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GTPase	guanosine triphosphatase
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
Ig	immunoglobulin

KLF	Kruppel-like factor
LTD	long-term depression
LTP	long-term potentiation
LTR	long terminal repeat
LV	lentivirus
MAG	myelin-associated glycoprotein
MAP1B	microtubule associated protein 1B
MIP	maximum intensity projection
MOI	multiplicity of infection
mTOR	mammalian target of rapamycin
NgR1	Nogo receptor 1
NO	Nitric oxide
NPY	Neuropeptide Y
NT-3	neurotrophin 3
OMgp	oligodendrocyte myelin glycoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PGK	phosphoglycerate kinase
PNG	peripheral nerve graft
PNN	perineuronal net
PNS	peripheral nervous system
PTEN	phosphatase and tensin homologue
PTPσ	protein tyrosine phosphatase sigma
ROI	region of interest
RPTP	receptor protein tyrosine phosphatase
SCI	spinal cord injury
SEM	standard error of the mean
SpH	synaptopHluorin
Syn-GFP	synaptophysin-GFP
VAMP2	vesicle-associated membrane protein 2
VSV-G	vesicular stomatitis virus G
WFA	*Wisteria floribunda agglutinin*
WPRE	woodchuck post-transcriptional regulatory element
YFP	yellow fluorescent protein

Publications arising from this work

Publications

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Abstracts

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CHAPTER 1

General Introduction

1 Introduction

Traumatic spinal cord injury (SCI) is a significant clinical problem. It is estimated that 2.5 million people worldwide live with the consequences of SCI, which include permanent neurological deficits in motor, sensory and autonomic function. Most patients exhibit a degree of spontaneous recovery after injury, but the majority are still left with permanent disabilities and no effective treatment for spinal injury currently exists. Nevertheless, optimism exists within the research community due to the emergence of therapies that have shown great promise preclinically, with the demonstration of functional improvements in experimental SCI models using a number of treatments. These potential future repair strategies will not be described in detail here, but have been extensively reviewed (Schwab, 2002; Fehlings and Baptiste, 2005; Bradbury and McMahon, 2006; Thuret et al., 2006; Donnelly and Popovich, 2008).

Following traumatic SCI two types of pathological process occur, consisting of primary and secondary events (Fehlings and Baptiste, 2005). Primary injury includes the physical forces acting on spinal cord tissue, such as compression, contusion or shear forces, and is not amenable to therapy. Secondary, reactive changes are triggered by the primary mechanical trauma and include processes such as blood-spinal cord barrier breakdown, disturbed ionic homeostasis, cellular dysfunction and inflammatory and immune changes (Dumont et al., 2001; Gris et al., 2008). These insidious, destructive pathophysiological changes are responsible for much of the tissue degeneration following injury and have been the focus of intensive research into neuroprotective mechanisms that aim to maximally preserve function of remaining tissue after the primary trauma (Hall and Springer, 2004). A number of pharamacological agents are currently in clinical trials including riluzole, a neuroprotective agent that prevents

glutamate release, and fampridine, a specific blocker of potassium channels on demyelinated axons (Hayes et al., 2004; Rabchevsky et al., 2011).

1.1 Promoting regeneration of injured axons

Despite the best efforts to contain the secondary effects of SCI, a significant degree of functional impairment will remain due to damage sustained by spinal neurons and axons. Interruption of axonal pathways leads to a total or partial disconnection of spinal segments below the lesion from supraspinal centres, which leads to a debilitating loss of voluntary control (Schwab and Bartholdi, 1996). A considerable research effort has focussed on stimulating the elongation of injured axons, with the aim of inducing them to regenerate beyond the lesion and reconnect with target neurons by forming functional synapses. Experimental approaches aiming to promote regeneration of injured axons broadly fall into two categories: enhancing the intrinsic ability of neurons to regenerate and neutralisation of inhibitory influences. In contrast with the robust regeneration of peripheral nerves, frank regeneration does not normally occur in the central nervous system (CNS) and it was long believed that this was an immutable state (Cajal, 1928). Pioneering experiments by Aguayo in the early 1980s questioned the dogma of the impossibility of CNS regeneration by showing that several hundred damaged CNS neurons would grow into a peripheral nerve graft (Richardson et al., 1980; David and Aguayo, 1981). These results indicated that, when presented with a growth-promoting microenvironment, injured CNS axons could regenerate. These findings led to the identification of growth-promoting factors found in the periphery and growth-inhibitory factors found in the CNS, which is discussed in more detail below.

1.1.1 *Enhancing the intrinsic regenerative ability of neurons*

Peripheral sensory neurons residing in the dorsal root ganglia have the unusual property of possessing two axons: one projecting through a peripheral nerve and the other projecting centrally, to the spinal cord. Some of these axons reach the dorsal column nuclei. This configuration lends itself to the study of peripheral versus central regeneration. Following injury to the peripheral branch of a sensory neuron, many transcriptional changes take place within these cells and a robust regenerative response ensues, whereas axon damage to the axon residing in the CNS does not result in any regeneration. However, when a conditioning lesion is applied to a peripheral nerve, central branches of primary sensory neurons exhibit an enhanced regenerative response (Richardson and Issa, 1984; Neumann and Woolf, 1999), suggesting that the failure of axotomoised CNS neurons to express growth-promoting genes contributes to their poor regenerative capability. Approaches to increase the regenerative ability of CNS neurons include the manipulation of signalling molecules such as cAMP that have been found to determine the intrinsic growth properties of CNS neurons (Qiu et al., 2002; Hannila and Filbin, 2008), decreasing the activity of the PTEN/mTOR pathway (Park et al., 2008; Liu et al., 2010), inhibiting the activity of the small GTPase RhoA (Ellezam et al., 2002) and promoting KLF4-mediated transcription (Moore et al., 2009). Additionally, the application of neurotrophins, such as BDNF and NT-3, has been found to remarkably increase the regenerative ability of CNS neurons (Bradbury et al., 1999; Ramer et al., 2000; Lu and Tuszynski, 2008). However, none of these manipulations alone is capable of inducing long range regeneration.

1.1.2 *Enhancing regeneration by targeting myelin*

Another important difference in regeneration between injured peripheral and central axons is the presence of inhibitory molecules after SCI, preventing regeneration beyond the injury site. This theory was first proposed in the 1980s when it was found that the injured CNS is a source of molecules that potently inhibit neurite outgrowth (Berry, 1982). A series of seminal papers followed, identifying CNS myelin as an important negative regulator of neurite outgrowth (Caroni and Schwab, 1988b). CNS myelin contains multiple proteins with growth inhibitory activity; the best studied of these is Nogo-A, which is taken to be the most potent myelin inhibitor (Cafferty et al., 2010). It has been reported that the application of neutralising antibodies against Nogo-A renders CNS tissue a more permissive substrate *in vitro* (Caroni and Schwab, 1988a; Savio and Schwab, 1989; Chen et al., 2000; Prinjha et al., 2000; Oertle et al., 2003). The application of Nogo-A antibodies *in vivo* has also been reported to promote regeneration, plasticity and functional recovery in animal models of SCI (Schnell and Schwab, 1990; Bregman et al., 1995; Maier et al., 2009), as has Nogo receptor blockade (GrandPre et al., 2002; Li and Strittmatter, 2003; Li et al., 2004; Li et al., 2005a; Freund et al., 2006). However, subsequent experiments using triple knockout mice lacking the three dominant axons growth inhibitors in myelin (Nogo, MAG and OMgp) proved controversial, with conflicting evidence from different laboratories (Silver, 2010). A thorough study using multiple lesion paradigms to study both regeneration and sprouting failed to show any significant regeneration through or beyond the lesion and only a slight increase in sprouting, which was not accompanied by behavioural improvement (Lee et al., 2010). At the same time, a different laboratory reported robust sprouting and regeneration in triple knockout mice, accompanied by remarkable behavioural recovery (Cafferty et al., 2010). This latter study used a single dorsal hemisection model of SCI, which is vulnerable to sparing of corticospinal tract (CST)

axons in the lateral funiculus or at the base of the dorsal columns and could lead to the false appearance of regeneration (Steward et al., 2003). Nevertheless, a human anti-Nogo-A antibody (ATI355) has completed a Phase I clinical trial, with no deleterious side effects observed (www.clinicaltrials.gov/ct2/show/NCT00406016), although efficacy has not yet been tested.

1.1.3 *Enhancing regeneration by targeting the glial scar*

A second inhibitory obstacle for regenerating axons is the glial scar, a dense collection of reactive astrocytes that seal the wound and protect surrounding tissue from cellular damage (Faulkner et al., 2004). The reactive astrocytes of the glial scar produce a range of molecules that are inhibitory to axonal growth (Silver and Miller, 2004). A prominent inhibitory family of molecules is the chondroitin sulphate proteoglycans (CSPGs), many of which are strongly inhibitory to neurite outgrowth *in vitro* (McKeon et al., 1991; Friedlander et al., 1994; McKeon et al., 1995; Smith-Thomas et al., 1995). The most common approach to mitigating CSPG-mediated inhibition is the use of a bacterial enzyme, chondroitinase ABC (ChABC) to cleave the glycosaminoglycan (GAG) chains from the core protein of the molecule (Prabhakar et al., 2005), thereby removing their inhibitory potential. This approach has been applied to many CNS injury models and has promoted regeneration of injured axons *in vitro* and *in vivo* (Smith-Thomas et al., 1995; Davies et al., 1999; Crespo et al., 2007). Following experimental SCI and ChABC treatment, regeneration of axons from different neuronal populations including motor (Bradbury et al., 2002; Iseda et al., 2008; Garcia-Alias et al., 2009), and sensory fibres (Bradbury et al., 2002; Yick et al., 2003; Cafferty et al., 2007; Shields et al., 2008) has been observed. Functional recovery has often accompanied these observations. A member of the receptor protein tyrosine

phosphatase (RPTP) family, RPTPσ, has recently been found to be a receptor that interacts with CSPGs, signalling neuronal inhibition (Shen et al., 2009). The loss of RPTPσ has been reported to accelerate axonal regeneration in the periphery of adult mice (McLean et al., 2002; Thompson et al., 2003) and has led to improved axonal regeneration after optic nerve injury (Sapieha et al., 2005). Similarly, following SCI axonal growth into the lesion after dorsal column crush injury is markedly enhanced by loss of RPTPσ (Shen et al., 2009), as is CST regeneration beyond a dorsal hemisection (Fry et al., 2010). However, the contribution of regeneration to the functional recovery seen in these animals is unknown, and the distances regenerated by axons are typically modest. In the early studies using ChABC, functional recovery was often attributed to the regeneration of fibres seen using anterograde or retrograde labelling, but it is possible that plasticity in other, untraced spinal systems were at least partly responsible for the improvements seen. Evidence for plasticity as a mechanism underlying the effects of ChABC is discussed below.

1.1.4 *Cellular transplantation for SCI*

In humans most spinal injuries result from blunt trauma and are contusive in nature, featuring a fluid-filled cyst or cavity surrounded by a rim of spared tissue (Norenberg et al., 2004). This large cavity represents an extremely hostile environment and a considerable obstacle for regenerating axons to negotiate. Attempts to counter this problem include the development of grafting and cellular transplantation therapies, which provide physical support across the lesion, as well as trophic support. Substrates that have been used to bridge lesion cavities include peripheral nerve, foetal cell or Schwann cell grafts, which provide a physical scaffold for elongating axons (Reier et al., 1988; Paino and Bunge, 1991; Cheng et al., 1996; Raisman, 1997). An important

role of transplanted cells is their considerable ability to enhance neuronal survival, regeneration and repair by secreting neuroprotective factors (Jones et al., 2001; Teng et al., 2006; Glazova et al., 2009). Specialised cells have also been used to combat the dysmyelination of axons that occurs after injury, including oligodendrocyte precursor cells and Schwann cells, that ensheath growing axons (Barnabe-Heider and Frisen, 2008; Rossi and Keirstead, 2009).

Clearly, SCI is a multifactorial problem including an unfavourable growth-inhibitory environment (Silver and Miller, 2004; Xie and Zheng, 2008), glial and inflammatory reaction (Popovich and McTigue, 2009), neuronal cell death and axonal disruption (Schwab and Bartholdi, 1996), insufficient stimulation of axonal growth by guidance cues (Giger et al., 2010) and an intrinsic failure of mature CNS neurons to activate regenerative transcriptional programs (Sun and He, 2010). The individual approaches described above will be limited in their ability to achieve CNS repair, as each experimental treatment will tackle only one of the many facets of this complex problem. Evidence from studies using combinatorial approaches is promising and reviewed thoroughly elsewhere (McCall et al., 2012).

1.2 Beyond regeneration

Promoting regeneration of the CNS after injury by targeting the lesion site has been the focus of much work for over a century, but the neurological significance of the regeneration observed experimentally remains unclear (Illis, 2012). There are several considerations that should be noted: the proportion of regenerating axons is small, the distance regrown by axons is modest and the contribution of regeneration to recovery is unknown. In one of the most impressive examples of CNS regeneration, *PTEN*

knockout mice exhibited axonal regeneration of up to 3mm beyond a complete spinal cord crush (Liu et al., 2010), but the authors report that many axons fail to penetrate the lesion site. Clearly, if the goal of regenerative research were for re-growing axons to reconnect with their original targets there remains a huge hurdle to achieving this goal, especially as in humans many thousands of neurons would have to regenerate tens of centimetres to reach their target neurons. There is more reason for optimism regarding the small numbers of regenerating axons, as it has been shown many times that even a small percentage of spared or regenerated axons can foster functional recovery (Bradbury and McMahon, 2006). The issue of connectivity of regenerated axons remains elusive. Ultrastructural evidence for synapse formation by regenerating axons has been described in several studies (Alto et al., 2009; Lu et al., 2012) and immunohistochemical visualisation of the immediate early gene c-fos provides a strong indication of connectivity. Firm electrophysiological evidence exists for the functional regeneration of sensory neurons following dorsal rhizotomy (Ramer et al., 2000; Steinmetz et al., 2005). Following SCI, there is an interruption of connections and the goal of most therapeutic strategies is to re-establish the connection between supraspinal centres and their target neurons below the lesion, either directly or indirectly. Direct reconnection can be provided by axonal regeneration, whereas indirect reconnection occurs via an alternative, multi-synaptic pathway and this compensatory anatomical rearrangement is known as plasticity. Evidence for regeneration has been provided in experimental models involving complete spinal cord transection (e.g. Fouad et al., 2005), but where the injury is incomplete it is difficult to separate the contributions of plastic and regenerative effects. The majority of human SCI is contusive in nature, with some degree of neural tissue sparing even in the most severe injuries (Kakulas, 1987; Tuszynski et al., 1999) and imaging evidence from a rat contusion model has shown that grey and white matter sparing is predictive of behavioural and electrophysiological

recovery (Kim et al., 2012). This remaining tissue presents a potentially more tractable target than for interventions aimed at promoting functional recovery after SCI compared to achieving true regeneration.

1.3 Neuronal Plasticity

The mature CNS was once thought to be hard-wired but changes in neural organisation are now known to be possible. The term 'neuronal plasticity' describes two main phenomena. Synaptic plasticity is the activity-dependent change in the efficacy of existing synapses. This form of plasticity is prominent during development when many connections are made, but experience determines which are maintained and which are pruned; it also underlies learning and memory formation. Anatomical plasticity is the structural, adaptive reorganisation of neural pathways and occurs after injury to the CNS.

1.3.1 Origins of the concept of neuronal plasticity

William James first linked the modifiability of behavioural habits and the strengthening of specific brain pathways by repeated use (James, 1890). He suggested that, as with footpaths, repeated use of neural paths would reinforce them. Tanzi and Lugaro built upon these ideas, proposing that localised synaptic facilitation was necessary for learning skills and forming mental associations (reviewed in Berlucchi and Buchtel, 2009). This view is remarkably aligned with the modern view of plasticity, especially in view of the fact that until Sherrington in 1897 there was no concept of the synapse (Berlucchi and Buchtel, 2009). At this time a clear theory of mental association was put forward by Lugaro: a coincidence of activity in two neurons leads to a newly formed

association between them (Lugaro, 1898). Although he was probably not the first to use the term neuronal plasticity, Cajal's theory of 'cerebral gymnastics' was very influential. He proposed that mentation was a product of plasticity, such that continuing stimuli would lead to an increase in neuronal connections (DeFelipe, 2006).

Plasticity was then largely ignored for several decades until the publications of Konorski (1948) and Hebb (1949), who brought the subject back into fashion with the publication of *The Organisation of Behaviour* (Hebb, 1949). Hebb favoured the concept of a reinforcement of existing synapses and changes attributed to this form of plasticity are sometimes referred to as 'Hebbian'. Evidence supporting his view that coincident activity leads to strengthened connections was provided by the discovery of long-term potentiation (Bliss and Lomo, 1973; Sejnowski, 1999). Cajal favoured the formation of new connections as a mechanism explaining plasticity and evidence supporting this theory was provided some years later by the discovery that dendrite branching and spine density increase when animals are exposed to enriched environments (Volkmar and Greenough, 1972).

1.3.2 *Regeneration, sprouting and plasticity*

So far interventions that aim to achieve re-growth of injured axons have been considered. However, this is just one form of growth response that occurs after injury. Before we consider the role of spontaneous and experimentally induced plasticity in CNS repair, it is important to define the axonal growth responses that occur after injury. It is now recognised that axonal growth after injury is a dynamic continuum, consisting of related phenomena (reviewed in Cafferty et al., 2008a). At one end of the spectrum is 'true' regeneration, which is the re-growth of an injured axon from its severed end.

Axons growing in this way rarely elongate more than a millimetre in the adult mammalian CNS, but can grow considerably more following experimental intervention (e.g. Schnell and Schwab, 1990). At the other end of the spectrum is synaptic plasticity, which occurs on the scale of microns and includes changes to spine density and dendritic branch morphology (Tan and Waxman, 2012). This form of growth involves biochemical change and anatomical alterations that are small but may nonetheless have significant biological consequences. Between these two extremes of the scale is the phenomenon of sprouting, which occurs on the scale of millimetres and can either be collateral (i.e. growth from the intact proximal segment of an injured axon) or sprouting stimulated in intact axonal fibres that are in proximity to injured fibre tracts, but undamaged themselves (Cafferty et al., 2008a). These are not clearly delineated terms and distinguishing sprouting and regenerating axons is not straightforward. However, it is important to understand the differences between such terms, if we are to correctly describe the effects of experimental therapies.

To study axonal growth after injury the classic method involved cutting a tract, labelling the axons of that tract and designating labelled fibres below the lesion as regenerated. However, if the lesion was incomplete, then spared fibres could be mistaken for regenerated fibres (Steward et al., 2003). When designing a study, the type of axonal growth expected will influence the choice of experimental injury model. For example, although a dorsal over-hemisection is a good model for studying CST regeneration, it is not a good model for CST sprouting and unilateral pyramidotomy would perhaps be more suitable (Lee and Zheng, 2012). Therefore, knowledge of the types of axonal growth stimulated by a treatment is important. Here, we define only growth of injured axons from the severed end as regeneration; this is often termed ‘true’ or ‘frank’ regeneration and was discussed above in the context of spinal cord repair.

Plasticity is a term encompassing the concepts of sprouting (both injured and intact fibres) and synaptic plasticity.

Cajal was the first to demonstrate synaptic replacement due to collateral sprouting in the dentate gyrus after a unilateral entero-rhinocortical lesion (Illis, 2012), although this finding was largely forgotten for a few decades, when Liu and Chambers (1958) reported collateral sprouting of intact afferents into regions of spinal cord denervated by dorsal root section. The first use of the term anatomical plasticity was by Raisman (1969) who demonstrated synaptic replacement due to collateral sprouting in septal nuclei at the ultrastructural level (Raisman, 1969). In contrast to axonal regeneration, sprouting is commonly observed spontaneously after SCI and several experimental treatments have been reported to enhance this response; these will be discussed in later sections.

As mentioned above, plasticity does not necessarily mean structural alterations but can include altered CNS physiology due to changes in synaptic input to cells (Illis, 2012). An important example is the unmasking of latent synapses when cells are deprived of their normal input (Wall, 1977) and begin to respond to new inputs that would not cause responses in intact animals.

1.3.3 *Spontaneous Plasticity after Spinal Cord Injury*

Most human spinal cord injuries are incomplete and accompanied by a degree of spontaneous functional recovery, which can be substantial (Wernig and Muller, 1992; Dietz et al., 1998; Fawcett et al., 2007). Experimental SCI in animal models is also followed by a period of improvement in locomotion and on behavioural tasks (Rossignol et al., 1999; Weidner et al., 2001; Bareyre et al., 2004; Courtine et al., 2005).

This improvement in function was originally attributed to recovery from spinal shock (Holaday and Faden, 1983; Hiersemenzel et al., 2000), a period following SCI that, in humans, is characterised clinically by areflexia or hyporeflexia (1-3 days) and the gradual return of reflexes and the development of hyper-reflexia, over weeks and months (Ditunno et al., 2004). The initial phase of spinal shock is thought to be due to a loss of descending excitatory influence to spinal neurons, as well as loss of descending inhibition of spinal inhibitory neurons (Ditunno et al., 2004), but it has become increasingly apparent that neural plasticity underlies many of the changes in reflex activity and function during this dynamic period. For example, it has been shown that two major components underlying improved function in humans were compensation and neural plasticity (Curt et al., 2008). Compensation refers to an improvement in function without an accompanying change in neurological deficit, e.g. an adapted movement strategy, as opposed to plasticity, which involves a change in neural circuitry.

1.3.3.1 Cortical reorganisation after nervous system injury

Although axonal regeneration rarely, if ever, occurs spontaneously in the adult mammalian CNS, spontaneous reorganisation of the adult CNS neuronal connections occurs readily after neural injury. Early studies focussed on cortical reorganisation following nerve injury in the periphery, where it was shown that denervated areas of cortex are initially silenced, but are invaded over time by neighbouring, intact afferents (Kaas, 1991). This has been shown to occur in the cortices of adult primates (Merzenich et al., 1983b; Merzenich et al., 1983a; Pons et al., 1991) and rats (Donoghue et al., 1990; Sanes et al., 1990). Cortical neurons appear to compete to occupy space and several mechanisms have been suggested to underlie this ‘invasion’ of unoccupied cortical space, including the unmasking of horizontal connections. Features of the

sensory environment are processed vertically across cortical layers, but horizontal projections between neighbouring cortical representational areas allow context-dependent processing of this information and the balance of excitation and inhibition promotes competition (Adesnik and Scanziani, 2010). In addition, experience-dependent activity between neighbouring cortical neurons, such as LTP, may be responsible for the recognition of patterns of representation and represent a spatial substrate for competition (Hess and Donoghue, 1994, 1996). The removal of intracortical inhibition after nerve injury has also been shown to contribute to alterations in connectivity (Jacobs and Donoghue, 1991; Brasil-Neto et al., 1993), especially in the light of the finding that intracortical inhibitory neurotransmitter levels can be related to sensory experience (Hendry and Jones, 1986). Finally, changes in cortical synapse number and morphology have been described during motor map reorganisation (Kleim et al., 2004; Kim et al., 2008).

1.3.3.2 Cortical reorganisation after SCI

Spontaneous reorganisation of motor and sensory cortical areas has also been observed following incomplete SCI. Numerous electrophysiological studies have shown remodelling of the somatosensory cortex following sensory afferent damage in the dorsal columns of the spinal cord (Kaas et al., 2008). For example, following partial dorsal column section, intact afferents initially continue to activate their normal target territories, but after some weeks these target areas become enlarged (Jain et al., 1997). Similarly, functional recovery of hand function has been shown to parallel re-emergence of the hand cortical map after unilateral CST lesion (Schmidlin et al., 2004). Rodent models of SCI have used electrophysiological, anatomical and imaging techniques to show this expansion of intact cortex into neighbouring, deprived areas.

Electrophysiological approaches include intracortical microstimulation (Fouad et al., 2001; Martinez et al., 2009) and recording cortical activation in response to peripheral stimuli (Ghosh et al., 2010), which has shown that cortical changes occur within minutes of injury (Aguilar et al., 2010). Imaging studies have also shown the dynamic nature of cortical maps, with techniques such as voltage-sensitive dye (Ghosh et al., 2009; Ghosh et al., 2010) and functional magnetic resonance imaging (fMRI) (Endo et al., 2007; Nishimura and Isa, 2009; Ghosh et al., 2010) being employed. These studies showed an expansion of the representation of the unimpaired limb (Endo et al., 2007; Ghosh et al., 2009) and the importance of both ipsi- and contralateral cortical changes in recovery (Nishimura and Isa, 2009). More recently similar changes have been observed in humans using positron emission tomography (Bruehlmeier et al., 1998; Curt et al., 2002a), fMRI (Curt et al., 2002b) and transcranial magnetic stimulation (Brasil-Neto et al., 1993; Endo et al., 2009).

1.3.3.3 Spinal reorganisation after SCI

Although less research has focussed on spontaneous plasticity after injury at the spinal level, recent observations suggest that reorganisation of spinal neural circuitry is considerable (Raineteau and Schwab, 2001). Studies have largely considered three main fibre systems: descending tracts from supraspinal centres, the propriospinal system of interneurons and the spinal locomotor pattern generator. Following experimental SCI, sprouting of injured and uninjured spinal axons occurs spontaneously in response to distant synaptic loss. For example, sprouting of lesioned hindlimb CST neurons into cervical spinal cord has been shown following a distant (thoracic) SCI and these axons become rewired in order for injured hindlimb CST axons to innervate the neurons of a spared body part (Fouad et al., 2001; Ghosh et al., 2010). Similarly, disruption of the

major, dorsal component of the CST has led to extensive spontaneous sprouting of the minor ventral and lateral CST components at the lesion site (Weidner et al., 2001; Bareyre et al., 2005) and this sprouting parallels functional recovery (Weidner et al., 2001). Spontaneous sprouting has long been suggested to contribute to functional recovery (Murray and Goldberger, 1974; Aoki et al., 1986) and it still believed that sprouting of CST fibres is important in mediating functional recovery after SCI (Jankowska and Edgley, 2006). As well as sprouting at and above the lesion site, a robust sprouting response of spared axons into the distal, denervated lumber segment of cord has been described, including spared descending serotonergic axons (Saruhashi et al., 1996) and ascending sensory afferents (Ondarza et al., 2003). Studies involving lesions of single tracts reveal that certain tracts can compensate for the loss of others. For example, the rubrospinal and bulbospinal tracts mediate functional compensation following the loss of the CST (Kennedy, 1990) and rats with localised damage to the lateral or ventral funiculi, which contain the reticulospinal and vestibulospinal tracts, exhibit similar functional recovery so presumably one tract compensates for the loss of the other (Loy et al., 2002).

The role of plasticity of the proriospinal system in mediating functional recovery was shown in an elegant study from the Schwab laboratory. Following a thoracic dorsal hemisection, injured CST fibres rostral to the lesion sprouted into the ipsilateral grey matter and made novel connections with propriospinal neurons, forming a route to bypass the injury and maintain a pathway for communication between corticospinal neurons and spinal motor neurons (Bareyre et al., 2004). The functionality of the newly formed connections was confirmed using both electrophysiology and trans-synaptic tracing, and improvements in hindlimb function were observed. Re-lesion of the CST rostral to the lesion reversed the functional improvements and electrophysiological

responses to cortical stimulation, suggesting that the rewiring of CST connections was necessary for mediating the functional improvement (Bareyre et al., 2004). Furthermore, the formation of relay circuits via reorganisation of descending and propriospinal connections can mediate recovery of treadmill locomotion, even if all descending pathways have been interrupted by staggered hemisections (Courtine et al., 2008). This ability of the spinal cord to remodel and form relay connections to bridge an injury site presents a different and possibly more achievable approach to treating CNS lesions, such as those found in stroke, multiple sclerosis and SCI.

The central pattern generator (CPG) is a circuitry within the spinal cord that is capable of generation the basic locomotor rhythm (Rossignol et al., 2006). The rhythmic, alternating patterns of excitation and inhibition are generated by the oscillatory properties of pacemaker cells coupled with excitatory interneurons (Grillner, 2003; Brocard et al., 2010). Following thoracic/cervical SCI an intact system of spinal circuits and sensory afferents remains to organise locomotion, entirely or partly cut off from supraspinal control. It has been shown that kittens (Forssberg et al., 1980) and adult cats (Barbeau and Rossignol, 1987; Belanger et al., 1996; de Leon et al., 1998b) can recover hindlimb locomotion following a T13 complete transection and several weeks of locomtor training. Sensory inputs are not required to produce the basic oscillatory rhythm but are critical in modulating and adapting locomotion to suit the environment. Afferent input from joints, skin and muscles interact with the CPG and dynamically shape the locomotor output (reviewed in Rossignol et al., 2006). The reliance of locomotion on sensory feedback is heightened after the loss of descending inputs and this was shown by experiments where animals underwent peripheral nerve injury before or after complete SCI (Rossignol and Frigon, 2011). If peripheral nerves were disrupted before injury, the recovery process was severely disrupted (Frigon and Rossignol,

2009). However, if an animal underwent spinal transection and locomotor training before the nerve injury, recovery did not suffer (Carrier et al., 1997), showing that sensory input alters reflexes in a desirable way during the training process. Considerable changes in spinal reflexes after SCI occur both spontaneously and due to activity-dependent processes, such as training (Cote and Gossard, 2003). Indeed, following incomplete SCI the novel appearance of evoked responses in rats after epidural stimulation (Lavrov et al., 2006) and humans after cutaneous nerve stimulation (Dietz et al., 2009) has been associated with recovery of locomotion. However, incomplete SCI presents a more complex picture, as there are several descending modulatory influences with access to spinal circuitry that may be spared. The reticulospinal and vestibulospinal tracts are important for the initiation of, and postural control during, locomotion. The CST and rubrospinal tracts are involved in the execution of more volitional, complicated tasks and goal-directed locomotion (Drew et al., 2004). The propriospinal system has also been shown to contribute to the recovery of volitional locomotion (Kato et al., 1984; Courtine et al., 2008). Importantly, connectivity from supraspinal centres is necessary for regaining voluntary control of locomotion, which is a desirable outcome for many spinal injured patients. The importance of compensation by intrinsic spinal circuitry has been investigated using a dual injury paradigm of an incomplete lesion followed several weeks later by a complete transection (Barriere et al., 2008; Barriere et al., 2010). Cats that undergo complete transection normally take several weeks and intensive training to regain treadmill locomotion, but those that had received an incomplete injury prior to the transection were able to walk within hours of the transection (Barriere et al., 2008; Barriere et al., 2010). These findings demonstrate the essential nature of an intact intrinsic spinal circuitry that is able to form new, adaptive configurations. Similarly, Basso et al. (1996) found that rats that underwent a spinal contusion 7 weeks prior to a

complete transection achieved better scores on a test of locomotor function than those that received transection only (Basso et al., 1996). Many studies of partial spinal lesions have attributed any observed functional improvements to effects mediated by descending tracts and have largely overlooked the potential importance of changes in spinal circuitry (Rossignol and Frigon, 2011).

1.3.4 *Promoting plasticity after SCI – rehabilitation*

Spontaneous plasticity after SCI parallels a functional improvement, independent of any therapeutic intervention, that leads to partial recovery. There is now considerable evidence that rehabilitative training can further promote this plasticity and harness it for useful functional changes (Fouad and Tetzlaff, 2012; Musienko et al., 2012). The potential of rehabilitative training to promote functional improvement after SCI was first shown using cats with complete spinal cord transection. After several months of intense treadmill training, cats regained weight-bearing locomotion and were able to walk on a treadmill for considerable periods of time (Lovely et al., 1986; Barbeau and Rossignol, 1987). The plasticity underlying these improvements was found to be activity dependent as improvements were observed only in tasks that the cats had been trained to perform. For example, cats that had been trained to stand were not able to step on a treadmill (Tillakaratne et al., 2002), showing that the isolated spinal cord had 'learned' a specific task in a 'Hebbian' manner. In this way, coordinated neuronal activity that is triggered by the repetitive movements during rehabilitative training can promote recovery after CNS injuries. It is likely that rehabilitation-induced plasticity is superimposed onto spontaneous changes. However, separating the contribution of the two types of plasticity in rodent models of SCI is not straightforward because rodents tend to self-train in their home cages because they remain mobile, even after a severe injury (Fouad et al., 2000; Kuerzi et al., 2010).

Locomotor training has been used successfully to improve motor function below the level of SCI, both clinically and in laboratory experiments (Edgerton et al., 2006). For example, daily treadmill training of rats with thoracic transection has been shown to reinforce spinal locomotor circuitry (Ichiyama et al., 2008). The success of locomotor training in achieving weight-bearing locomotion in the laboratory has led to the translation of activity-based rehabilitation into the clinic, where it is used as therapy for humans with partial SCI (Dietz and Harkema, 2004; van Hedel and Dietz, 2010). This approach has yielded improvements in ambulatory function in patients with incomplete SCI (Wernig and Muller, 1992; Dietz et al., 1994; Barbeau et al., 1999; Behrman and Harkema, 2000). Indeed, rehabilitative training alone is currently the most successful treatment for SCI, as has been shown by a clinical trial which reported that 92% of subjects regained the ability to walk at a functional speed within 3 months (Dobkin et al., 2007). However, locomotor training does not result in improvements in locomotion in patients with severe SCI (Dietz, 2009). This has been suggested to be due to the depressed state of spinal networks, such that there is insignificant activity in neuronal networks remaining to be trained (Dietz et al., 1994; Harkema, 2008). Strategies to change the physiological state of spinal circuits have been developed to increase neuronal activity and thereby facilitate stepping and weight bearing. These include electrical spinal cord stimulation and the use of pharmacological agents to promote a permissive state (reviewed in Musienko et al., 2012).

Physical rehabilitation may improve functional outcomes in several ways (Fouad and Tetzlaff, 2012). Electrophysiological studies have shown that motor training can affect the neurophysiological characteristics of motoneurons. For example, one study showed that there was a strong correlation between stepping behaviour after training and the

amplitude of synaptic inputs to motoneurons, as well as the action potential afterhyperpolarisation amplitude (Petruska et al., 2007). Physical rehabilitation has also led to rearrangements of cortical maps, such that the area representing the trained limb expands (Girgis et al., 2007). In addition, the up-regulation of growth factors following physical rehabilitation has been described (Maier et al., 2008), particularly brain derived neurotrophic factor, BDNF (Vaynman and Gomez-Pinilla, 2005; Ying et al., 2005).

Despite the promising results described above, several studies in recent decades have shown that rehabilitative training can be a double-edged sword (Tetzlaff et al., 2009). Cats that had been trained to weight-bear learned to stand for long periods, but performed worse than untrained cats on a treadmill; in contrast cats trained to walk could stand less well than untrained cats (De Leon et al., 1998a, b). Similarly, rats with cervical spinal lesions that had been trained to perform a reaching task deteriorated in their performance on an untrained locomotor task, the horizontal ladder (Girgis et al., 2007). In addition, animals trained in a task specific manner to perform reaching and grasping had improved manual dexterity (when combined with CSPG digestion) but animals treated with ChABC and given general rehabilitation in an enriched environment performed even worse on the reaching task than untreated control animals (Garcia-Alias et al., 2009). Encouragingly, one study has reported that the combination of training for both ladder walking and grasping leads to improvements in both trained tasks (Dai et al., 2009). Taken together, these results suggest that considerable care should be taken when planning rehabilitation so that certain tasks are not gained at the expense of others, which may become lost due to lack of practice.

1.3.5 *Promoting plasticity after SCI – targeting myelin*

1.3.5.1 Myelin and plasticity in the naïve brain

The role of myelin-associated inhibitors in restricting plasticity after SCI is well described and will be discussed below. More recently it has been established that these molecules play a physiological role in the intact CNS by limiting anatomical, experience-dependent and activity-dependent plasticity.

A classic example of CNS plasticity is the shift in ocular dominance of binocular cortex, whereby deprivation of one eye leads to an increased representation of the other in the visual cortex (Crair et al., 1998). This shift can only be induced during the critical period of development (Crair et al., 1998), a period of heightened plasticity, during which neuronal properties are highly prone to modification, especially through experience (Berardi et al., 2000). The end of this activity-driven period of heightened plasticity in the visual system occurs weeks to months after birth in rodents and at about 5 years of age in humans (Hensch, 2004). Its closure coincides with an increase in myelination in the visual cortex, suggesting a role for myelin in the loss of ocular dominance shifts as adulthood is reached (McGee et al., 2005). Nogo plays a role in restricting ocular dominance plasticity in an NgR1-dependent manner, as shown by experiments with Nogo-A and NgR1 knockout mice. Loss of either protein leads to a persistence of ocular dominance shifts well into adulthood, clearly indicating a role for the Nogo-Ngr1 axis in the closure of the critical period (McGee et al., 2005). Another recently described high affinity receptor for inhibitory myelin components, Paired Immunoglobulin-like Receptor B (Atwal et al., 2008), also limits ocular dominance plasticity (Syken et al., 2006). Thus, experience-dependent processes in the mature, intact CNS are regulated by myelin components.

1.3.5.2 Inhibition of myelin

The earliest evidence for the limitation of collateral sprouting by myelin components came from experiments employing unilateral CST lesions at the level of the medullary pyramid, which completely lesion one tract, while leaving the other projection intact. The first study showing sprouting of intact CST fibres after pyramidotomy administered an anti-Nogo antibody, IN-1, post-lesion and saw significant sprouting of CST fibres in the developing spinal cord (Schwab and Schnell, 1991). Anti-Nogo treatment has also led to CST sprouting in the mature rat spinal cord of both intact and injured axons, which were visualised by anterograde labelling of the lesioned CST, and this was accompanied by recovery of forelimb function (Thallmair et al., 1998; Z'Graggen et al., 1998). Pyramidotomy has also been shown to lead to CST sprouting in the brainstem, at the level of the pontine nucleus (Blochlinger et al., 2001). CST plasticity has also been studied in the context of experimental stroke models where, at the level of the brainstem or spinal cord, one CST tract is damaged and the other left intact by a distant injury. Sprouting of the intact CST into the denervated side was seen at the level of the midbrain and spinal cord and was associated with improved functional recovery (Papadopoulos et al., 2002; Lee et al., 2004). In addition to rodent studies, anti-Nogo antibody treatment has been shown to improve primate hand function following a cervical unilateral hemisection (Freund et al., 2006, 2009) accompanied by an increased number of midline crossing sprouts (Freund et al., 2007). These studies showed that CST axons sprout across the midline, leading to bilateral innervation of spinal cord or brainstem targets. Of course, the CST is not the only tract to undergo plastic changes following suppression of Nogo-A using antibodies. For example, intact rubrospinal tract fibres have been reported to sprout into the ventral horn of the spinal cord after bilateral pyramidotomy (Raineteau et al., 2002). Serotonergic fibres have also been shown to sprout into lumbar spinal cord after

thoracic SCI and re-establish connections after application of an anti-Nogo antibody (Mullner et al., 2008; Maier et al., 2009). In addition to antibodies, other treatment types that block the Nogo receptor (NgR1) or its ligands have also been employed to similar effect. NgR1 blockade using the antagonist NEP1-40 leads to sprouting of various descending tracts after SCI, including serotonergic and CST fibres, and this has often accompanied functional improvements (GrandPre et al., 2002; Li and Strittmatter, 2003; Cao et al., 2008). Targeting the NgR1 co-receptor, LINGO-1, has also led to sprouting of both rubrospinal and CST axons (Ji et al., 2006). Moving away from pharmacological or immunotherapy, transgenic studies using knockout mice lacking myelin components such as myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp) and various Nogo isoforms (Nogo-A, -B and -C) have also been carried out. Following SCI in MAG/OMgp/Nogo-A knockouts enhanced sprouting has been observed, primarily due to the loss of Nogo-A (Cafferty et al., 2010; Lee et al., 2010). However, the functional significance of this finding is controversial as improved locomotion was found by one laboratory (Cafferty et al., 2010) but not by another (Lee et al., 2010). The pharmacological and immunotherapy studies targeting Nogo or NgR1 were primarily carried out in rats, whereas the transgenic studies utilised mice. However, the magnitude of improvements seen in performance on behavioural tests was comparable between studies using Nogo-A or NEP1-40 (e.g. GrandPre et al., 2002; Lee et al., 2004) and those using Nogo-A knockout mice (Cafferty et al., 2010). Deletion of Nogo-A/-B has been shown to lead to a significant increase in sprouting of both injured and uninjured axons in mouse models of SCI of differing severity (Kim et al., 2003; Simonen et al., 2003; Cafferty and Strittmatter, 2006). In one of these studies, the increase in midline-crossing sprouts correlated with functional recovery (Cafferty and Strittmatter, 2006). Similarly, in mice lacking NgR1 that had undergone thoracic hemisection collateral sprouting of the raphespinal tract was observed and this was

responsible for the improved locomotor recovery in knockouts versus heterozygotes (Kim et al., 2004).

These studies provide evidence that myelin-associated factors inhibit collateral sprouting, suggesting that they restrict non-regenerative growth. As the sprouting phenomenon often coincides with functional recovery, it is likely that compensatory anatomical growth is aiding the development of indirect connections between the brain and the spinal cord, thereby mediating behavioural improvements.

1.3.6 *Promoting plasticity after SCI – targeting CSPGs*

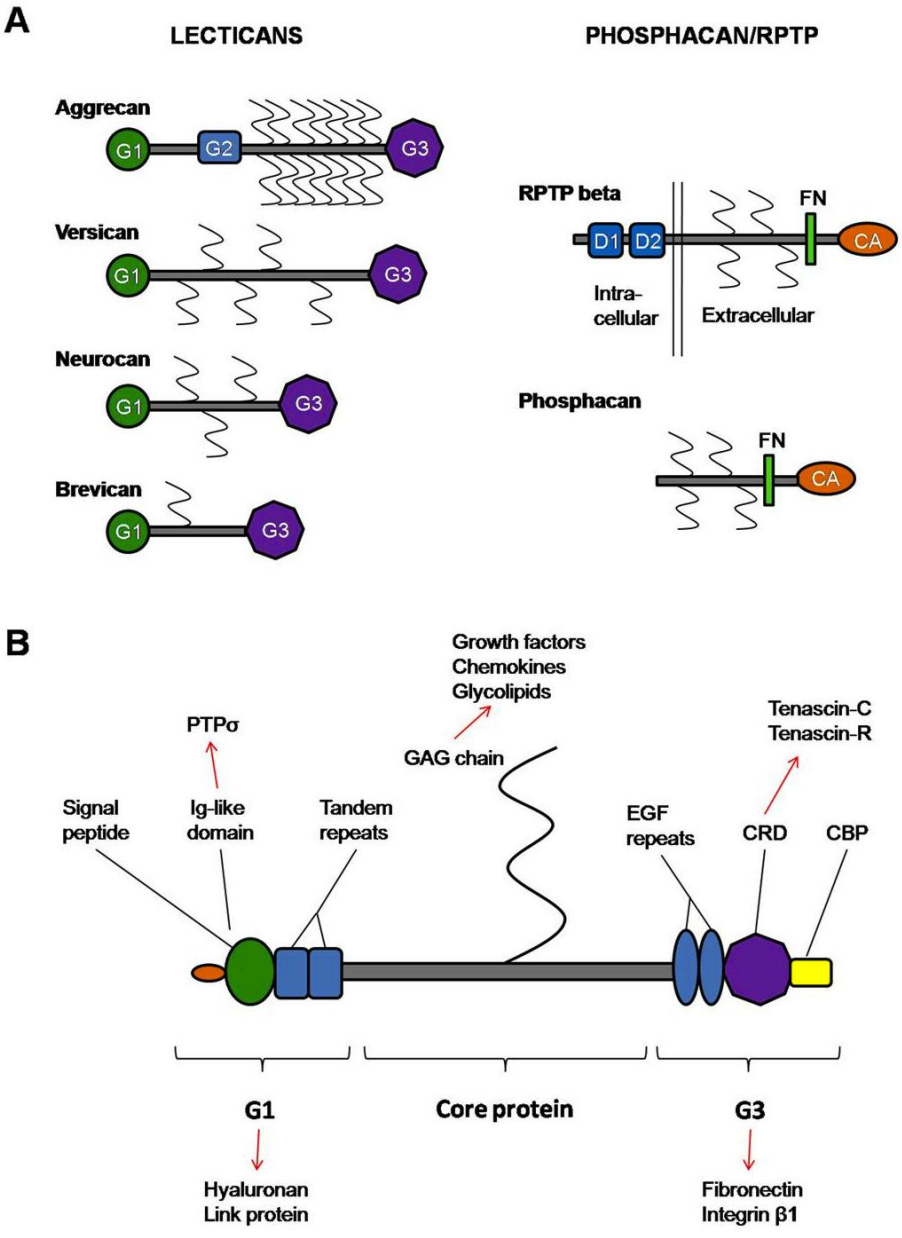
1.3.6.1 Structure and interactions of CSPGs in the CNS

CSPGs are major components of the CNS extracellular matrix (ECM). They are a divergent group of molecules that are grouped according to the characteristics of their core proteins, which contain different domains (Fig. 1.1). The specific protein moieties of the core protein affect the interactions and signalling capabilities of the particular CSPG family member. The core protein is covalently linked to linear polysaccharide chains, known as chondroitin sulphate glycosaminoglycans (CS-GAGs; Kjellen and Lindahl, 1991), which vary in number between 1 to >100 per core protein molecule. The disaccharide sugar units that make up CS-GAGs can be sulphated by chondroitin sulphotransferases (Sugahara and Mikami, 2007). The resulting heterogeneous and precise sulphation patterns determine the interactions of CSPGs with other molecules and encode functional information (Gama et al., 2006). The variation in structure of CSPGs helps to explain their wide range of molecular interactions, including ECM proteins and growth factors. CSPG interactions with other ECM proteins are essential for the formation of the perineuronal nets (PNN), which are structures of condensed matrix that form around the cell bodies and proximal dendrites of certain classes of

neuron (Celio et al., 1998). Other major PNN components include the tenascins (Bruckner et al., 2000), hyaluronan (Yasuhara et al., 1994), and link protein (Bekku et al., 2003), all of which are known to interact with CSPG components of the PNN (Galtrey and Fawcett, 2007a). A summary of the interactions of lecticans, the most abundant CSPG family in the CNS, is shown in Fig. 1.1B. During CNS development, expression levels and sulphation patterns of CSPGs are tightly regulated (Cortes et al., 2009). The participation of CSPGs during the development is reviewed elsewhere (Oohira et al., 2000; Carulli et al., 2005; Sugahara and Mikami, 2007).

1.3.6.2 CSPGs in the visual system

As mentioned above, the phenomenon of ocular dominance plasticity involves the shift towards increased cortical representation of one non-deprived eye following deprivation of the other eye (Berardi et al., 2003). As the CNS matures, this shift is no longer possible. Evidence for the involvement of CSPGs in limiting cortical plasticity has been revealed by several studies investigating ocular dominance. Organisation of CSPGs into PNNs coincides with the end of this period of heightened plasticity (Pizzorusso et al., 2002). Dark rearing prolongs the critical period and delays CSPG accumulation into PNNs in the visual cortex (Guimaraes et al., 1990; Hockfield et al., 1990; Lander et al., 1997; Pizzorusso et al., 2002), indicating that PNN formation in the visual cortex is activity-dependent. Digestion of PNN CSPGs in the visual cortex using ChABC led to an ocular dominance shift following monocular deprivation in adult rats (Pizzorusso et al., 2002), showing that the mature ECM inhibits experience-dependent plasticity and that CSPG degradation can reactivate cortical plasticity. In a further study, rats were subjected to monocular deprivation prior to the end of the critical period, until adulthood. This led to an irreversible shift in ocular dominance and a reduction in visual acuity in the affected eye, as in humans with amblyopia. Treatment of these


A
LECTICANS
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Aggrecan
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Versican
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Neurocan
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Glycolipids
PTPσ
GAG chain
Tenascin-C
Tenascin-R
Signal
peptide
Ig-like
domain
Tandem
repeats
EGF
repeats
CRD
CBP
G1
Core protein
G3
Hyaluronan
Link protein
Fibronectin
Integrin β1

Fig. 1.1: Schematic representation of the most common CNS CSPGs and their environmental interactions. (A) Lecticans are composed of two globular N- and C-terminal domains (G1 and G3, respectively) flanking a core protein region where GAG chains attach via serine residues. Aggrecan has an additional G2 domain adjacent to the G1 domain. (B) Phosphacan is a splice variant of the full-length receptor-type protein-tyrosine phosphatase β (RPTPβ) that lacks the two intracellular tyrosine phosphatase domains (D1 and D2). Both of these have an N-terminal carbonic anhydrase domain (CA), CS-GAG attachment regions and a fibronectin type III domain (FN). (C) Schematic showing molecular components of the G1 and G3 domains of a lectican, indicating target areas for interactions with binding partners and interactions with other ECM molecules (red arrows). CRD, Carbohydrate recognition domain; CBP, complement binding protein; EGF, epidermal growth factor; Ig, immunoglobulin. From Bartus et al., 2011.

animals with ChABC led to a complete recovery of ocular dominance, visual acuity and spine density (Pizzorusso et al., 2006). Similar results have been described in mice lacking the cartilage link protein Crtl1, which had disrupted PNN formation and elevated levels of ocular dominance plasticity persisting into adulthood (Carulli et al., 2010). Together, these studies show that disrupting PNNs by targeting one of their major components can lead to persistent plasticity and that an intact PNN is required to restrict plasticity in the mature CNS.

1.3.6.3 CSPGs in memory

Evidence for a role of CSPGs in CNS plasticity has also come from memory-related models, including long-term potentiation (LTP) and long-term depression (LTP) in the hippocampus (Dityatev and Schachner, 2003), as well as from studying fear memories (Quirk et al., 2010).

Fear conditioning, in adult animals, is induced by pairing an ambient stimulus with a painful stimulus, which results in the formation of a permanent memory that is resistant to erasure. Extinction training can generate an inhibitory memory capable of suppressing the conditioning effect, but only temporarily (Quirk et al., 2010). In contrast, extinction training can completely erase the fear memory if performed during the postnatal period. The amygdala is involved in fear conditioning and contains PNNs, which have recently been suggested to play a role in the developmental regulation of fear conditioning (Gogolla et al., 2009). The appearance of PNNs in the amygdala coincides with the end of the developmental period when extinction can result in permanent erasure of fear memories, suggesting a role for PNNs in preserving these memories. Furthermore, ChABC digestion of PNN CSPGs in the amygdala of adult mice led to the permanent erasure of fear memories using extinction, without affecting

the acquisition of the conditioning effect (Gogolla et al., 2009). These results suggest that the fear memories of adult animals are actively protected by the presence of PNNs.

The hippocampus has been extensively studied for its plasticity in response to brief periods of altered input, LTP and LTD (Bliss and Collingridge, 1993; Malenka and Bear, 2004). The role of PNNs in this form of plasticity has been investigated using hippocampal slice cultures. The removal of CS-GAG chains by exposing the slice to ChABC was found to reduce both LTP and LTD (Bukalo et al., 2001). Transgenic approaches allow the modification of PNNs by removal of single components. PNNs are less prominent in brevican deficient mice and the maintenance of hippocampal LTP is severely impaired (Brakebusch et al., 2002). Neurocan knockout mice have normal-looking PNNs and LTP development, but the maintenance of late-phase LTP is impaired (Zhou et al., 2001). The divergent effects of neurocan and brevican absence on LTP suggest that PNN components have differing roles in the development and maintenance of LTP.

1.3.6.4 CSPGs in synaptic stability – possible mechanisms

Once neural circuits have been formed by experience-dependent processes their connections must be stabilised to maintain performance. Due to its known inhibitory effects on structural rearrangements and axonal growth, the ECM is believed to play an important role in long-term synaptic stabilisation and the maintenance of synaptic networks (Dityatev et al., 2010a). This is illustrated by examples from the visual system and memory-related models in the preceding sections. However, the molecular mechanisms of this inhibition remain elusive. Putative mechanisms include (i) signalling pathway regulation, (ii) a physical barrier for the lateral diffusion of membrane molecules, and (iii) regulation of neuronal excitability.

Growth inhibitory molecules, including CSPGs, signal through epidermal growth factor receptor (EGFR), RhoA and ROCK (Koprivica et al., 2005). Activation of this signalling pathway leads to growth cone collapse and ChABC may promote structural plasticity by blocking the activation of this pathway (Borisoff et al., 2003; Sivasankaran et al., 2004). In addition, ChABC treatment leads to an increase in the phosphorylated form of extracellular regulated signal kinase 1 (ERK1), an enzyme involved in excitatory synaptic plasticity (Carter et al., 2008). Direct interactions of CSPGs with specific receptors also inhibit axonal outgrowth and induce growth cone collapse. It has recently been shown that CSPGs bind with high affinity to the protein tyrosine phosphatase σ (PTPσ) transmembrane receptor (Shen et al., 2009; Fry et al., 2010) and prevent their oligomerisation, leading to the inhibition of axonal growth (Coles et al., 2011).

Transmembrane proteins, including neurotransmitter receptors, can move laterally in the membrane but do not move freely as they are limited by intracellular proteins and ECM molecules. The fast exchange of AMPA receptors between synaptic and extrasynaptic sites allows the modulation of synaptic transmission (Heine et al., 2008) but the presence of compartments formed by PNNs limits this lateral diffusion (Frischknecht et al., 2009). In this study, enzymatic removal of a key PNN component, hyaluronan, led to increased receptor exchange and changes in short-term plasticity (Frischknecht et al., 2009).

CSPGs are enriched around axons and have been implicated in the compartmentalisation of axonal proteins. For example, brevican is enriched at the axon initial segment and perisomatically (John et al., 2006). It also accumulates at the nodes of Ranvier of large diameter myelinated axons and is likely to be responsible for

organisation of other nodal ECM components (Bekku et al., 2009). Experiments using mice lacking the versican splice variant V2 have shown that paranodes form normally and nodal ion channels cluster appropriately, but the nodal ECM formation is largely impaired (Dours-Zimmermann et al., 2009). Experiments using cultured hippocampal interneurons have shown that CSPGs accumulate around axons in an activity-dependent way and their removal with ChABC leads to increased neuronal excitability (Dityatev et al., 2007). This finding suggests that CSPGs are involved in the regulation of neuronal activity. Supporting evidence for this hypothesis is provided by the observation that CS-GAGs are involved in the regulation of local diffusion of Ca^{2+} in the brain (Hrabetova et al., 2009). Extracellular Ca^{2+} concentration plays a critical role in regulating neuronal excitability and synaptic transmission and it is possible that CSPG-mediated limitation of its diffusion may regulate these features.

1.3.6.5 ChABC treatment promotes plasticity after SCI

As discussed in previous sections, axonal sprouting and new circuit formation following SCI parallels spontaneous recovery (Weidner et al., 2001; Bareyre et al., 2004; Courtine et al., 2008; Rosenzweig et al., 2010). As CSPGs play a significant role in restricting plasticity in the mature CNS, CSPG modification presents an attractive approach to enhance the spontaneous plasticity after injury and potentially improve functional recovery. The most common strategy used to target CSPGs is their digestion with ChABC, which removes their inhibitory CS-GAG side chains and has been shown by many studies to have positive effects on functional recovery after experimental SCI, when used alone or in combination (Bradbury and Carter, 2011). It has been convincingly demonstrated that ChABC treatment promotes regeneration and neuroprotection (Bradbury et al., 2002; Fouad et al., 2005; Crespo et al., 2007; Carter et

al., 2008; Tester and Howland, 2008; Carter et al., 2011). Here, the beneficial effects of CSPG modification on promoting plasticity after SCI will be discussed.

CSPGs are present in the uninjured spinal cord in the form of PNNs, which encapsulate many spinal neurons and restrict their growth and plasticity. After SCI, a glial scar forms and CSPG levels rapidly increase, which makes the environment intensely inhibitory to axonal growth (Silver and Miller, 2004; Busch and Silver, 2007). Thus, CSPGs are exerting inhibitory effects at two levels: the PNNs surrounding surviving neurons and glial scar-associated CSPGs, providing two targets for anti-CSPG therapies to promote plasticity following SCI.

ChABC injection can render the uninjured CNS more adaptive, as shown by experiments in the visual system (Berardi et al., 2004). ChABC has also been shown to increase structural plasticity in the form of sprouting in denervated CNS regions, following distant injury. For example, following partial retinal injury, undamaged retinal axons exhibited significantly increased sprouting into the superior colliculus with ChABC and BDNF treatment (Tropea et al., 2003). In the partially denervated brainstem, following cervical dorsal column lesion, ChABC treatment led to increased sprouting of uninjured forelimb primary afferents into denervated territory (Massey et al., 2006). This sprouting was visualised anatomically, using cholera toxin tract tracing, and the functional connectivity of these sprouts was shown using electrophysiological receptive field mapping. This study is significant because it demonstrates that sprouting can be directly linked to a functional improvement. Spared primary afferents have also exhibited significantly increased sprouting in the dorsal horn that was denervated by multiple cervical rhizotomies of the C5, C6, C8 and T1 dorsal roots (Cafferty et al., 2008b). This sprouting of spared C7 fibres was accompanied by improved sensory

behavioural function and electrophysiological changes, which showed the novel formation of connections between second order spinal neurons and spared primary afferent fibres in ChABC treated animals. Increased plasticity in the denervated spinal cord following peripheral nerve injury has also been observed by increased immunostaining for a marker of sprouting axons, phosphorylated MAP1B, after spinal microinjection of ChABC (Galtrey et al., 2007). Sprouting was accompanied by behavioural improvements in grip strength and skilled reaching after nerve injury paradigms that resulted in misguided regeneration in the periphery. This study shows that central plasticity can compensate for inaccurate motor and sensory reinnervation in the periphery. Plasticity of both intact and injured fibres within the spinal cord following SCI was first shown by Barritt et al., (2006). A cervical dorsal column lesion was employed and intrathecal ChABC treatment was found to promote sprouting of intact serotonergic fibres and peptidergic primary afferents caudal to the lesion (Barritt et al., 2006). Unlesioned fibres of the dorsolateral CST have also been shown to sprout into spinal grey matter regions containing digested CSPGs following disruption of the major, dorsal CST component (Garcia-Alias et al., 2009). This ChABC induced sprouting accompanied a partial recovery of motor function. The above studies have all been carried out in rodent SCI models but it has also been shown that ChABC treatment of a cervical hemisection in a larger mammal, the cat, led to marked serotonergic sprouting and improved functional recovery (Tester and Howland, 2008). These studies show that compensatory plasticity by spared fibres is restricted by CSPGs and may play an important role in promoting useful function after SCI and ChABC treatment.

Sprouting of injured fibres after SCI has also been described. Following a cervical dorsal column lesion that almost entirely abolished the dorsal CST, tract tracing showed that injured CST fibres sprouted rostral to, within and caudal to the lesion (Barritt et al.,

2006). Long distance regeneration of CST fibres would be required for reconnection with their original targets, which is rarely seen using any experimental treatment and only in a small number of fibres. The formation of a relay circuit by sprouting CST fibres connecting with the propriospinal system to bypass the lesion has been suggested as an attractive alternative to long distance regeneration (Courtine et al., 2008) and could be enhanced by ChABC.

1.3.6.6 Plastic changes after combinatorial treatments using ChABC

SCI is a severe, multifactorial problem with many concurrent problems and mitigation of inhibitory CSPGs is just one of the many possible targets for potential therapies. By combining experimental treatments that target distinct problems, it has been possible to achieve better results than by using therapies in isolation (Lu and Tuszynski, 2008). One strategy has been to combine a peripheral nerve graft (PNG) with ChABC to make the tissue surrounding the graft more permissive. Houle et al., (2006) used a PNG to bridge a C3 hemisection and applied ChABC at the distal site of apposition with the cord. This study showed a synergistic effect of using both approaches together, with animals performing significantly better on behavioural tests of forelimb use. After cutting the PNG some of the behavioural effects lingered, suggesting that local reorganisation of spinal circuitry induced by ChABC may have contributed to the functional improvement (Houle et al., 2006). Recently, a similar study used a C2 hemisection model to investigate the effect of PNG combined with ChABC treatment on recovery of respiratory function (Alilain et al., 2011). This study showed that ChABC alone could cause sprouting of intact serotonergic fibres and a limited restoration of diaphragm function investigated using electrophysiological techniques. However, when the ChABC and a PNG were used in combination, diaphragmatic recovery was remarkable (Alilain et al., 2011). Further, transection of the PNG led to a

transient increase in tonic diaphragm activity, indicating considerable remodelling of spinal circuitry. As this was not observed in animals that had received the PNG alone, the role of CSPGs in restricting functional reorganisation is confirmed. Other combinatorial strategies include the combination of ChABC with growth factors or cell transplants. One study using the growth factor NT-3 in combination with ChABC showed that reinnervation of the deafferented gracile nucleus could be promoted by either treatment alone, but using both together led to a superior synergistic effect (Massey et al., 2008). Karimi-Abdolrezaee et al. (2010) also used a combination therapy approach and showed that ChABC is an important part of the therapy. This study combined ChABC, a cocktail of growth factors and a transplant of neural progenitor cells to promote the survival of graft cells and encourage functional plasticity. The results of this study were that both CST remodelling and a functional improvement were achieved only when all three of the therapies were combined. Importantly, when ChABC was left out of the combination no increase in neuroanatomical sprouting was observed, underlining the key role for CSPG digestion in promoting plasticity (Karimi-Abdolrezaee et al., 2010). A recent convincing example of increased plasticity consisted of a lateral hemisection and a combination treatment consisting of ChABC, NT-3 and the NR2D subunit of the NMDA receptor (Garcia-Alias et al., 2011). In this study electrophysiological techniques were used to demonstrate the formation of a relay pathway around the hemisection, allowing novel polysynaptic connectivity of lateral white matter fibres with ipsilateral lumbar motoneurons. This plasticity was accompanied by sprouting of reticulospinal fibres, which was only present in ChABC treated animals, and accompanied a behavioural improvement (Garcia-Alias et al., 2011). Only those animals that received the full combination exhibited these plastic phenomena, suggesting that the growth-promotion and synaptic modulation of the treatments act in concert to produce a synergistic effect.

1.3.7 *Combination of plasticity-enhancing therapies and rehabilitation*

Ultimately, an SCI therapy is likely to combine a regenerative or plasticity-promoting pharmacological treatment with training to direct meaningful connectivity. This principle is reminiscent of the situation during development of the nervous system, where many connections are made, but only those that are used are maintained and stabilised, whereas unused connections are pruned (Murakami et al., 1992). In principle a plasticity-promoting treatment could facilitate the formation of numerous non-specific connections, which will then be refined and translated into functional recovery by rehabilitative training. As all SCI patients receive some form of rehabilitation as part of their care, any pharmacological treatment would have to work in conjunction with training (Dobkin and Havton, 2004). Thus, pre-clinical studies assessing this combinatorial approach are vital. Recently a number of studies have addressed this issue and have combined training regimes with growth factors, ChABC (Garcia-Alias and Fawcett, 2012) or anti-Nogo treatment (Starkey and Schwab, 2012).

Boyce and colleagues (2007) performed complete spinal transections in adult cats and assessed their ability to regain quadrupedal stepping on a treadmill. Animals received either a transplant of NT-3 and BDNF expressing fibroblasts into the lesion site, daily treadmill training, or both combined. The authors reported that untrained animals that received neurotrophins only performed as well as animals that had undergone daily treadmill training. Animals that received a combination of training and neurotrophins did not exhibit further improvements, except on one outcome measure (Boyce et al., 2007). As axonal growth through the transplant was observed, it is likely that the neurotrophins were affecting spinal circuits below the level of the injury (de Leon,

2007). As release of neurotrophins is one mechanism by which training exerts its effects, this may explain the lack of additive effect of the two treatments.

A second study examined the effects of daily treadmill training combined with anti-Nogo antibody treatment after an incomplete thoracic SCI in adult rats (Maier et al., 2009). Animals that received the antibody alone showed a good recovery of coordinated locomotion as shown by detailed kinematic analysis. Rats that had received treadmill training only also recovered coordinated locomotion, although kinematic parameters indicated a more abnormal gait than antibody treated rats. Surprisingly, rats that received both interventions performed worse than both other groups, with very dysfunctional stepping. The authors suggest that the differing mechanisms of improvement induced by the two treatments may interfere with each other, or that the timing of the commencement of training was inappropriate (Maier et al., 2009). The latter explanation is supported by the finding that, when using the same injury and anti-Nogo treatment, delaying the onset of training to four weeks after injury resulted in a very good functional outcome (Marsh et al., 2011).

A third investigation from the Fawcett laboratory investigated the effects of ChABC treatment and task specific training, either alone or in combination, on rats' ability to reach for sugar pellets after a dorsal column injury (Garcia-Alias et al., 2009). Neither ChABC treatment nor reaching training alone led to any recovery of skilled forelimb function. However, administering both treatments simultaneously led to a significant effect, with rats recovering the ability to retrieve around two-thirds as many pellets as they had pre-lesion. As improved hand function is a high priority for patients with spinal cord injuries (Anderson, 2004), this study indicates an exciting opportunity towards developing combination treatments for patients. A cautionary element of the

study by Garcia-Alias et al. (2009) is the detrimental effect on forelimb reaching the authors observed when animals underwent general rehabilitation. Clearly, the competing and synergistic effects of pharmacological and rehabilitative therapies are not straightforward and further work will be required to elucidate the mechanisms underlying injury- and treatment-induced changes.

1.4 Aims of the Thesis

The work in this Thesis aims to firstly, use viral vector technology to deliver the enzyme chondroitinase ABC to the rodent spinal cord following peripheral or central nervous system injuries; secondly, to investigate the functional and anatomical changes associated with injury and treatment, with an emphasis on electrophysiology; and thirdly, to develop a novel tool for investigating plasticity in the nervous system by using viral vectors to label the synaptic terminals of neuronal populations both *in vitro* and *in vivo*.

The experimental findings are presented in the following Chapters:

Chapter 2: Chondroitinase ABC promotes plasticity of spinal reflexes following peripheral nerve injury

Chapter 2 uses electrophysiological techniques to investigate the effect of two different levels of inaccurate peripheral reinnervation on low and high threshold spinal reflexes. Following lentiviral delivery of ChABC to digest CSPGs, several indicators of reorganisation of central connections were observed. These included increases in the amplitude of low threshold polysynaptic reflexes, protection of a high threshold reflex, wind-up, from injury induced collapse and the novel appearance of wind-up responses in an extensor nerve, the radial. These results suggest that the application of a plasticity-

promoting treatment to the spinal cord allows reorganisation of central connections in response to inaccurate wiring in the periphery.

Chapter 3: Gene delivery of chondroitinase ABC promotes functional repair after spinal cord injury

Employing a thoracic contusion injury, Chapter 3 examines the functional outcomes after SCI and treatment with lentiviral ChABC. Functionally, *in vivo* electrophysiological testing reveals increased conduction across the lesion and plasticity of spinal reflexes below the lesion, and animals also performed better on a behavioural test of sensorimotor function. Anatomical examination showed a significant amelioration of lesion pathology and changes in the appearance of the glial scar of treated animals. Together, these results indicate that CSPG digestion can promote repair and functional improvement after traumatic SCI.

Chapter 4: Developing viral vectors for the study of anatomical plasticity in the nervous system

Chapter 4 describes the development lentiviral vectors expressing synaptic proteins and their use *in vitro*. Application of different viral vectors *in vivo* revealed that an adenoviral vector of AAV2/5 serotype produced better transduction than lentiviral vectors. Thus, AAV2/5 was used to express a synaptic protein with pH-sensitive GFP tag, synaptopHluorin, in two neuronal populations in vivo, the CST and sensory neurons. The terminals of labelled axons were brightly labelled, allowing quantification of synaptic puncta.

CHAPTER 2

Chondroitinase ABC promotes plasticity of spinal reflexes following peripheral nerve injury

2.1 Introduction

2.1.1 *Peripheral nerve injury*

Peripheral nerve injury frequently has long-term debilitating consequences due to the loss of motor and sensory function conveyed by that nerve, as well as secondary consequences such as neuropathic pain (Jaquet et al., 2001). Despite refinement of surgical techniques for peripheral nerve repair, functional recovery is often only partial (Lundborg, 2003). This is in part due to poor specificity of target reinnervation by regenerating axons, which is known to be inaccurate when axons regenerate over long distances (Koerber et al., 1989; Valero-Cabre et al., 2004; Guntinas-Lichius et al., 2005b; Navarro et al., 2007).

In theory, it is possible that topographical errors in regeneration can be compensated for with appropriate reorganisation of central processing. However, such plasticity appears to be limited. It has been reported that children recover better than adults after surgical repair of a peripheral nerve (Rosen et al., 2000; Lundborg and Rosen, 2001; Jerosch-Herold, 2003). Studies comparing outcomes after nerve repair in adult or infant monkeys found that regeneration success was not different, even though functional outcomes were better in the younger group (Almquist et al., 1983). This suggests that the immature CNS has a greater capacity for central reorganisation.

2.1.2 *Central plasticity after peripheral nerve injury*

In adult mammals there is some evidence for central plasticity. For example, after cut and repair of the sciatic nerve regenerating sensory neurons lose normal topography (Fawcett and Keynes, 1990). When these sensory axons first regenerate to skin, dorsal horn neurons receiving sciatic input exhibit, as expected, very large and diffuse

receptive fields. However, with time, these receptive fields become focussed and approximately normal in size (Lewin et al., 1994). This reorganisation is blocked by spinal NMDA antagonists, suggesting an activity-dependent, Hebbian mechanism of functional reorganisation of sensory connections. However, such reorganisation is limited in extent. For instance, somatotopic reorganisation remains disrupted. Further, a number of studies have demonstrated that some spinal circuitry, for instance monosynaptic connections made by muscle spindle afferents with homonymous motoneurons cannot be respecified (Mendell and Scott, 1975; Lisney, 1983). Thus, despite the capacity for central changes following peripheral nerve injury, functional outcome remains poor.

2.1.3 *Enhancing plasticity with ChABC*

Strategies to enhance central plasticity may improve the outcome, for example modulating components of the CNS extracellular matrix. CSPGs have emerged as important regulators of plasticity (Kwok et al., 2008; Bartus et al., 2011). Their appearance in the spinal cord during development, in the form of PNNs, marks the end of the critical period of plasticity (Kalb and Hockfield, 1988, 1990; Takahashi-Iwanaga et al., 1998; Galtrey and Fawcett, 2007a) and mice lacking a PNN component, tenascin-R, show improved functional recovery after facial nerve injury and repair (Bruckner et al., 2000; Guntinas-Lichius et al., 2005a). Similarly, animals lacking Crtl1 link protein with attenuated PNNs have persistent anatomical plasticity in medullary sensory nuclei (Carulli et al., 2010). Furthermore, CSPGs have recently been shown to be involved in receptor exchange between synaptic and extrasynaptic sites (Frischknecht et al., 2009), the maintenance of contextualised fear memories (Gogolla et al., 2009) and the induction of hippocampal long-term potentiation and depression (Bukalo et al., 2001). These findings suggest that CSPGs are involved in stabilising synaptic connections

(Dityatev et al., 2010b). A commonly employed strategy for manipulating CSPGs is enzymatic removal of their inhibitory glycosaminoglycan side chains using the bacterial enzyme ChABC. This strategy has been shown to improve functional recovery after CNS injury, particularly in the spinal cord (Bradbury et al., 2002; Caggiano et al., 2005; Tester and Howland, 2008; Garcia-Alias et al., 2009) and there is a growing consensus that plasticity is at least partially responsible for this improvement (Barritt et al., 2006; Tester and Howland, 2008; Garcia-Alias et al., 2009; Alilain et al., 2011; Bradbury and Carter, 2011). In addition to promoting plasticity when delivered to the vicinity of an injury, ChABC has also been shown to reactivate plasticity in the intact CNS, when its delivery to the visual cortex allowed a shift in ocular dominance following visual deprivation during the critical period (Pizzorusso et al., 2002; Pizzorusso et al., 2006). CSPG digestion has also been shown to have a remarkable plasticity-promoting effect when ChABC is applied to the denervated brainstem (Massey et al., 2006) or spinal cord (Galtrey et al., 2007; Cafferty et al., 2008b).

Galtrey et al. (2007) used peripheral nerve injuries of varying severities to study the effect of central delivery of ChABC, together with daily training, on functional recovery. These injuries provided degrees of misguidance for the regenerating axons. Behavioural testing following treatment revealed significantly improved grip strength in animals that were severely impaired due to a misguided nerve injury paradigm; furthermore, animals that had undergone correct repair showed improvements in skilled forelimb function. However, the nature of changes in connectivity that might underlie such improved outcomes has not yet been investigated.

2.1.4 *Aims of the Chapter*

In the present Chapter electrophysiological techniques were used to investigate the effect of ChABC on spinal reflexes after peripheral nerve injuries of varying severities. The crossed nerve injury employed by Galtrey et al. (2007) involved crossing two forelimb flexor nerves. In contrast, here the more severe of the injury paradigms involved crossing a flexor nerve with an extensor, resulting in a greater degree of misguidance. Results show that the monosynaptic reflex between heteronymous muscles did not change after nerve injury, but polysynaptic connections of both myelinated and unmyelinated afferents were altered following application of a plasticity promoting treatment to the spinal cord. We propose that in this way inaccurate wiring in the periphery is compensated for by reorganisation of spinal circuitry due to increased permissiveness in the spinal cord environment.

2.2 Methods

2.2.1 *Surgical procedures*

Animals and experimental groups. 46 male Wistar rats were used in this study. There were 5 experimental groups: naïve (n=9), correct repair (n=7), crossed repair (n=8), correct repair treated with ChABC (n= 10) and crossed repair treated with ChABC (n=6). Of the groups with correctly repaired nerves, a subset of untreated (n=3) and ChABC-treated (n=6) rats were used exclusively for pain studies. The remaining rats were used for electrophysiology and assessment on a skilled behavioural test. The contralateral forelimb nerves of 8 ChABC-treated rats were used as treated, uninjured controls. An additional group of 6 naïve animals was used to investigate the time course of CSPG digestion following LV-ChABC injection (n=2 at 2 weeks; n=2 at 4 weeks; n=2 at 8 weeks).

Lentiviral vector. ChABC was delivered via an integrating, self-inactivating lentiviral vector, which is described in detail in a recent publication (Zhao et al., 2011). The vector was pseudotyped with VSV-G and produced using a second-generation system, as described previously (Naldini et al., 1996b; Hendriks et al., 2007). Expression of the ChABC gene was driven by the cytomegalovirus promoter. The ChABC gene inserted into the lentiviral vector was a modified version designed to optimise enzyme secretion from mammalian cells (Muir et al., 2010). Viral titre, obtained by a p24 ELISA assay, was 99μg/ml, corresponding to $6x10^5$TU/μl.

ChABC delivery and peripheral nerve injuries. All surgical procedures were performed in accordance with U.K. Home Office regulations (European Communities Council Directive of 24 November 1986 (86/609/EEC)). For recovery surgery, sterile precautions were used. Rats were anaesthetised with 60mg/kg ketamine and 0.25mg/kg medetomidine, diluted in saline and administered i.p. Body temperature was monitored

rectally and used to regulate a homeothermic blanket. A lentiviral vector expressing ChABC (LV-ChABC) was used in these experiments, delivered by intraspinal injection. Lentiviral vector mediated gene delivery to the CNS is highly efficient and not cytotoxic (Kordower et al., 2000; Abordo-Adesida et al., 2005; Abdellatif et al., 2006). Minimal, asymptomatic inflammation caused by lentiviral vectors is especially weak when using VSV-G coated vectors, as in the present study (Mazarakis et al., 2001). Rats were prepared for surgery by shaving and disinfecting the dorsal surface of the back over the scapulae. After skin incision the layers of muscles were dissected through to expose the lower cervical vertebrae. A partial laminectomy of the C5 vertebra was performed and gel foam soaked in 0.2% lignocaine was placed on the dura covering the dorsum of the spinal cord for two minutes, then a small incision was made in the dura using small spring scissors (Fine Science Tools) and lignocaine was reapplied for a further two minutes. A single injection of 1ul of LV-ChABC into the right C5 ventral horn was then performed using a glass capillary pulled to a diameter of about 20µm, connected to a 10µl syringe and driven by a microdrive pump (Harvard Apparatus) at 0.25µl/min. The injection coordinates were 1mm from the midline, first lowering the glass capillary 2mm, then raising it 500µm for the first two minutes of the infusion and raising it a further 500µm for the remaining two minutes of the infusion. The capillary was left in place for two minutes after the injection. Overlying muscle and skin were sutured in layers. 1mg/kg atipamezole hydrochloride subcutaneously was used to reverse the anaesthetic. Animals recovered in an incubator and received 0.05mg/kg buprenorphine postoperatively. A group of rats received LV-ChABC only and were sacrificed at 2 (n=2), 4 (n=2) and 8 (n=2) weeks to investigate the extent of CSPG digestion. All other rats were left for 3 weeks before undergoing nerve injury to allow lentiviral ChABC expression and during this period they underwent behavioural training.

For the peripheral nerve injuries, animals were anaesthetised as described above and incisions to the ventral skin and pectoralis major muscle of the right forelimb were made, exposing the brachial plexus near the axilla. The median and radial nerves were identified and underwent one of two types of lesion and repair (Fig. 2.1B): (i) both nerves were cut and repaired correctly by re-anastomosis (median to median and radial to radial); (ii) both nerves were cut and a crossover repair was performed (proximal median stump to distal radial stump and vice versa). Nerve transection was performed using spring scissors and repair entailed one or two stitches with 10-0 sutures (Ethicon, Ethilon^{TM}) to the epineurium. Surgery was concluded as described above and rats were left for up to 12 weeks in order for axonal regeneration to occur before animals underwent electrophysiological assessment. Animals recovered uneventfully and did not exhibit autotomy.

2.2.2 *In vivo electrophysiological recordings*

Preparation. Rats were terminally anaesthetised with an intraperitoneal injection of 1.25g/kg urethane (Sigma-Aldrich). Percutaneous electrodes in the left and right forelimbs recorded the electrocardiogram and body temperature was maintained around 37°C using a homeothermic blanket. A tracheotomy was performed and a tracheal cannula inserted. The brachial plexus of the right forelimb was exposed and the median, ulnar and radial nerves were dissected free from surrounding connective tissue. Skin flaps from the incision formed a pool, which was filled with paraffin oil. Whole nerve recordings, with the ulnar nerve mounted on silver wire hook electrodes, were used in these experiments. To confirm nerves had regenerated to the intended targets, the median and radial nerves were electrically stimulated in turn (100μs, 0.5Hz, 0-100μA), while mounted on silver wire hook electrodes proximal to the nerve repair site and the threshold for visible muscle contraction, as well as the type of contraction (flexor or

extensor), were noted. In all cases, correctly repaired median nerves produced flexor contraction and correctly repaired radial nerves produced extensor contraction. Median nerves that had been sutured into distal radial nerve stumps now produced extensor contraction and crossed radial nerves now produced flexor contraction. Nerves were then cut just proximal to the anastomosis site before reflex experiments were carried out (Fig. 2.1A).

Fast reflexes. The magnitude of monosynaptic and polysynaptic reflexes was determined in ulnar nerves upon stimulation of myelinated afferents in other brachial nerves. Electrical stimuli of increasing amplitude from 50 to 500μA, in 50μA steps, (100μs square wave pulse at 0.5Hz) were delivered from a constant current stimulator to the median or radial nerves. Ulnar nerve response to each stimulus was recorded. Recordings were captured with Scope software (ADInstruments). An average of 64 sweeps at 400μA was calculated online for each nerve and used to find the difference in amplitudes of reflexes evoked by median and radial nerve stimulation. This was achieved using software to calculate the absolute integral of any response between 1.5 and 3ms (monosynaptic) or 3 and 7ms (polysynaptic), regardless of whether a response is observed qualitatively (Fig. 2.2A). The 1.5-3ms window was chosen based on a calculation as follows: the afferent and efferent limbs of the reflex have a maximum conduction velocity of approximately 50m/second (Munson et al., 1997) and the conduction distance in both cases is 50mm. The quickest time for 2 synapses is 1msec (more for multi-synaptic responses). Therefore:

2msec conduction time + 1msec for 2 synapses = 3msec (fastest possible disynaptic response)

Thus, by our calculation, anything faster than 3msec has to be monosynaptic.

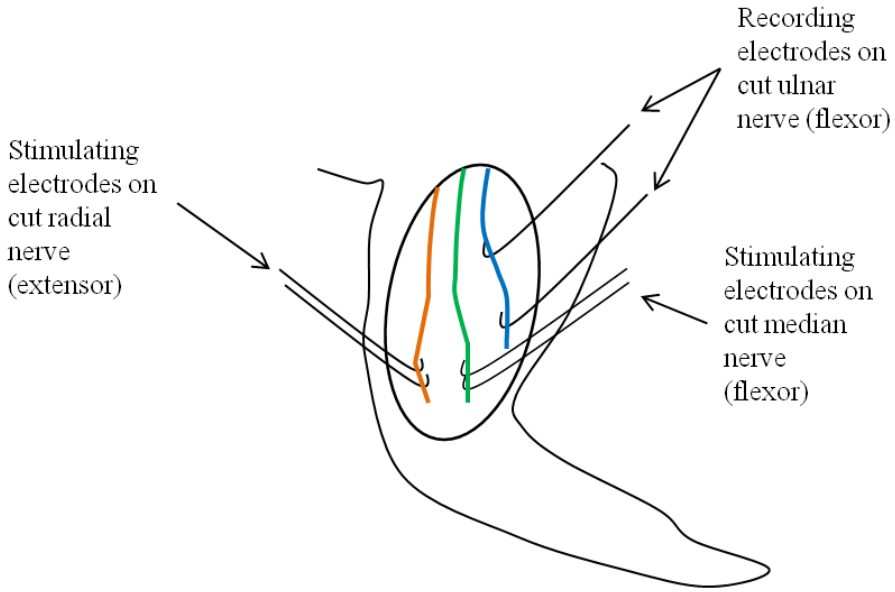
The 3-7ms interval was defined by the above calculation and the observation that polysynaptic responses rarely, if ever, appeared later than 7ms at low threshold stimulation.

Wind up. Peripheral nerve recordings can be used to study the phenomenon of wind up of motoneurons (Schouenborg and Sjolund, 1983; Woolf and Wall, 1986), a reflex reflecting spinal processing of nociceptive information. A train of 25 supramaximal stimuli was delivered to the median or radial nerves in turn at a stimulus intensity of 4mA (1ms square wave pulse at 0.5Hz). Recordings of ulnar nerve activity for 1 second after each impulse were captured using LabChart software (ADInstruments). Ulnar nerve activity was also recorded for 20 seconds prior to and 50 seconds after the stimulation period. Three trials were carried out for each nerve, with an interval of 5 minutes between trials to allow the ulnar nerve to return to its resting level of activity. A multi-unit recording of all spikes approximately 25% greater than the mean noise level was made, typically producing ~5 spikes per second under resting conditions. The number of spikes reaching this threshold was recorded for each second before, during and after the period of stimulation and plotted as a graph. To quantify wind-up from spike frequency plots, area under the curve analysis was performed. This value was then normalised to baseline nerve activity levels and input (the number of spikes discharged during the second following the first stimulus). The normalisation serves to control for differences in recording conditions and nerve excitability and is calculated as follows:

$$\text{Wind-up} = (\text{total spikes}) - ((\text{input} \times 25) - (\text{basal} \times 25))$$

Total spikes = sum of spikes recorded during the stimulating period. Basal = average number of spikes per second in the 20 seconds prior to the stimulating period. Two-way ANOVA was used to detect any statistical difference.

A


Recording electrodes on cut ulnar nerve (flexor)
Stimulating electrodes on cut radial nerve (extensor)
Stimulating electrodes on cut median nerve (flexor)

B

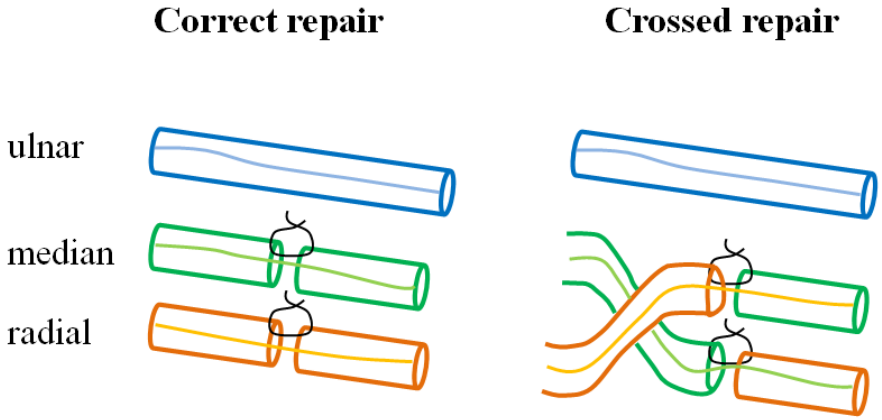

Correct repair
Crossed repair
ulnar
median
radial

Figure 2.1: Diagram illustrating electrophysiology and nerve injury paradigms. A: reflexes were measured by recording from the ulnar nerve, cut distally (blue, right). Recording electrodes were placed approximately 5mm apart. The median (middle, green) and radial (left, orange) nerves were stimulated in turn. Stimulating electrodes were placed distally, near the cut end of each nerve, approximately 2mm apart. **B**: two levels of severity of nerve injury were performed to produce graded levels of inaccurate reinnervation. For correct repair: the median (green) and the radial (orange) nerves were cut and immediately repaired and axons regenerated towards their original targets. For crossed repair the median and radial nerves were cut and the proximal stump of the median nerve (green), a flexor, was sutured to the distal stump of the radial nerve (orange), an extensor, so that regenerating median nerve axons (green lines) would reinnervate extensor radial nerve targets; the proximal stump of the radial nerve (orange) was sutured to the distal stump of the median nerve (green) so that regenerating radial nerve axons (orange lines) would reinnervate flexor median nerve targets.

2.2.3 *Behavioural assessment*

Skilled motor function. The staircase test, which provides a measure of skilled forepaw sensorimotor function, was used to assess reaching performance (Montoya et al., 1991). The use of this test after graded peripheral nerve injury has been described in detail elsewhere (Galtrey and Fawcett, 2007b). The apparatus (Campden Instruments Ltd.) consists of a central platform with seven steps either side. Each step holds one pellet (45mg, Research Diets Inc.) that the rat can reach for from the platform. The corridor containing the platform and steps is sufficiently narrow to prevent the rat reaching pellets on the right with the left paw and vice versa. Rats were trained daily for 3 weeks prior to nerve injury and those failing to reach the minimum criterion for inclusion in the study (2 pellets/side/day) were excluded from this behavioural test. Of the rats trained on this test (n=5 per group), 3 had to be excluded (1 each from repair untreated, crossed untreated and crossed treated). After injury, during thrice-weekly testing sessions the number of pellets successfully retrieved (7 pellets for each side per trial) was recorded. Each session consisted of two 5-minute trials at least 10 minutes apart. Mean scores per rat per testing week were calculated and baseline scores reflect the total number of pellets retrieved during the final week of training. Rats were on a restricted diet of 15g of food pellets per day during training and testing to encourage participation, and were weighed weekly to ensure that they maintained their body weight.

Sensory testing. Mechanical and thermal thresholds were ascertained to assess the effects of LV-ChABC and peripheral nerve injury on pain sensitivity. Pain behaviour after formalin injection was also assessed. Mechanical withdrawal thresholds were assessed using a dynamic plantar anaesthesiometer (Ugo Basile). Animals were placed in clear plexiglass cubicles on top of a metal grid and allowed to acclimate for 15 minutes. A computer-controlled stimulus was applied to the forepaw that applied a

linearly increasing force ramp. A cut-off of 50g was imposed to prevent any tissue damage. The force necessary to elicit paw withdrawal was recorded. Thermal hyperalgesia was assessed by applying a radiant heat source to the plantar surface of the forepaw and measuring the time taken for the rat to withdraw its paw (Hargreaves et al., 1988). The time taken to withdraw was measured three times for each paw and the mean latency was analysed. For both mechanical and thermal testing, the withdrawal threshold of each forepaw was calculated as the average of 3 consecutive tests with at least 5 minutes between each test. Measurements were taken on 3 separate days before injury and weekly thereafter. Formalin testing was performed at the end of the study (approximately 12 weeks after injury) as previously described (Dubuisson and Dennis, 1977). Briefly, 50µl 5% formalin in sterile saline was injected under the skin of the plantar surface of the right forepaw. The rat was then placed in a plexiglass observation chamber and the time spent licking/biting was measured over 45 minutes, in 5 minute bins.

2.2.4 *Tissue processing and immunohistochemistry*

C-4-S: For visualisation of digestion by virally expressed chondroitinase, rats were deeply anaesthetised using sodium pentobarbitone (80mg/kg, i.p.) and transcardially perfused with 200ml heparinised saline followed by 400ml paraformaldehyde (4% in 0.1M phosphate buffer). Spinal cords were dissected and post-fixed in 4% paraformaldehyde overnight at 4°C, then transferred into 20% sucrose for 48 hours at 4^{o}C. The tissue was blocked in OCT embedding compound for cryostat sectioning. 20µm thick sagittal sections were thaw-mounted onto Superfrost Plus slides and stored at -20^{o}C. To visualise GAG digestion sections were immunostained for chondroitin-4-sulphate (C-4-S), which is the digested stub of the CS-GAG chain. The frozen sections were incubated in the following (with three PBS washes between each step): normal

goat serum (10%, 30 minutes), mouse anti-C4S (1:5000, overnight; ICN Biochemicals), horse anti-mouse biotinylated secondary antibody (1:400, 90 minutes; Jackson ImmunoResearch), ABC reagent (1:250, 20 minutes; Vector Labs), biotinyl tyramide (1:75, 10 minutes; PerkinElmer Life Sciences) and ExtrAvidin TRITC (1:200, 2 hours; Sigma). Slides were coverslipped with Vectashield mounting medium (Vector Labs).

Wisteria floribunda agglutinin (WFA): this lectin binds to the terminal N-acetylgalactosamine residues of chondroitin sulphate chains (Young and Williams, 1985) and is reactive to PNNs in the CNS (Brauer et al., 1993). Slides were washed three times in PBS and incubated with TRITC conjugated WFA lectin (Patricell Ltd.) overnight at room temperature. Slides were washed in PBS and coverslipped as above.

2.3 Results

2.3.1 *Characterisation of spinal reflexes in intact animals*

Stretch reflex

A paradigm for studying a well-known spinal reflex, the monosynaptic stretch reflex between Ia afferents and motoneurons, was developed. A stereotypical fast wave in the ulnar nerve was observed upon median nerve stimulation. This wave had a latency of 1.83±0.025ms and appeared when a stimulation intensity of 100μA or higher was applied (Fig. 2.2A). As expected, it could not be elicited by stimulation of the extensor, radial nerve (Fig. 2.2B; $p<0.05$). Reflex magnitude was calculated by calculating the absolute integral and these data are shown in Table 2.1. As the latency of this fast response is consistent with a heteronymous connection of Ia spindle afferents in the median nerve exciting α-motoneurons that innervate synergist muscles in the ulnar nerve territory (Nelson and Mendell, 1978), this wave will hereafter be referred to as the monosynaptic reflex. Several responses of a longer latency (approx. 3.5-7ms) were also observed, this time after either median or radial nerve stimulation (Fig. 2.2A). The amplitude of these later responses evoked by stimulation of median and radial stimulation did not differ (Fig. 2.2C; $p>0.05$). The latency of these waves is consistent with spinal polysynaptic relays. Thus two types of spinal reflex that can be elicited between nerves of the brachial plexus were characterised and subsequently used to study plasticity following peripheral nerve injury.

Wind-up

In addition to the low threshold responses described above, ulnar nerve response to either median or radial nerve stimulation at supramaximal C-fibre intensities was investigated . Constant, repeated stimulation of the median nerve led to a successive

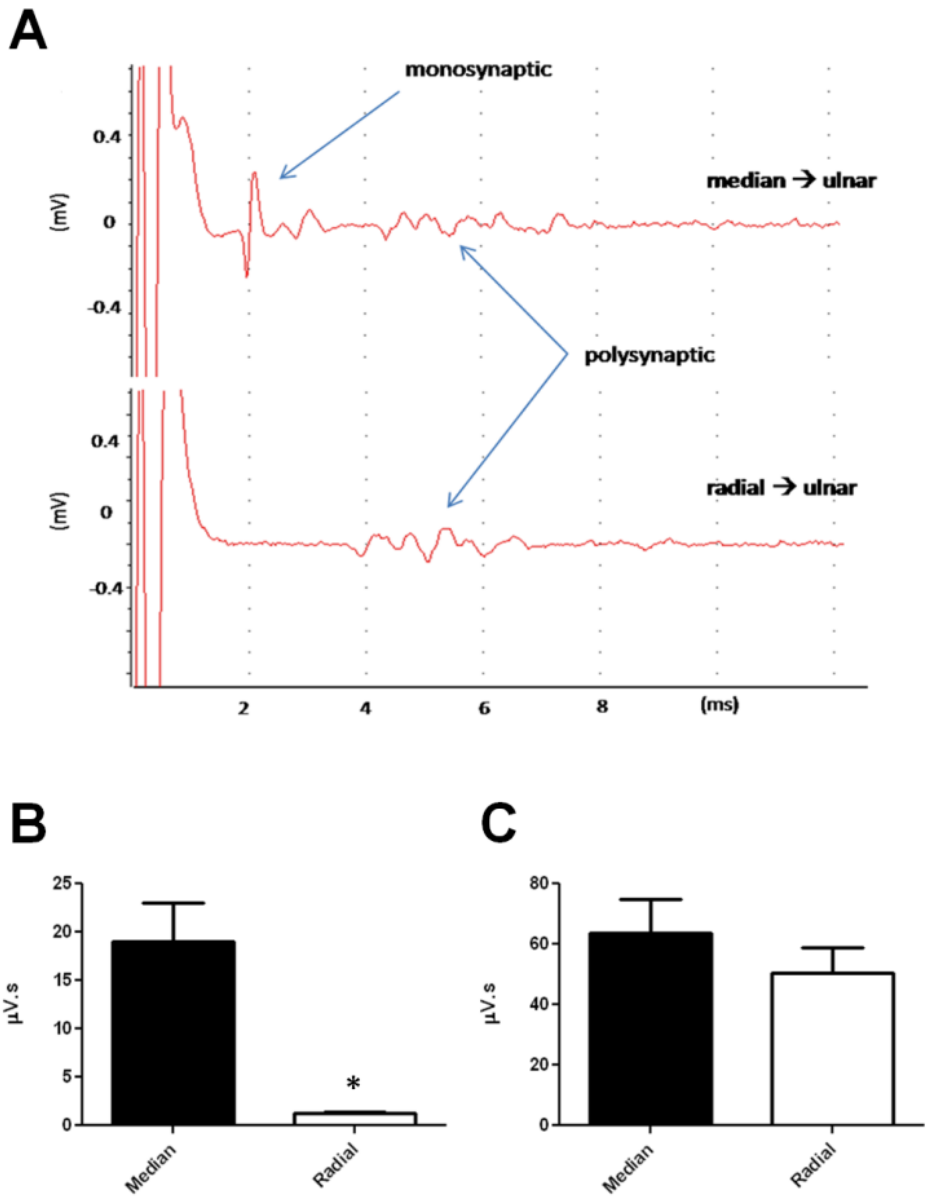

A
monosynaptic
median → ulnar
polysynaptic
radial → ulnar
0.4
0
-0.4
(mV)
2
4
6
8
(ms)
B
μV.s
25
20
15
10
5
0
*
Median
Radial
C
μV.s
80
60
40
20
0
Median
Radial

Figure 2.2: Characterisation of low threshold spinal reflexes. A: shown are representative examples of whole ulnar nerve recordings after stimulation of the median nerve (top trace) or radial nerve (bottom trace). The traces shown are averages of 64 sweeps, while stimuli of 400µA amplitude and 100µs pulse width were delivered at 0.5Hz. A fast reflex of 1.5-3ms latency is observed after median nerve stimulation. Longer latency, polysynaptic reflexes are present in both traces at 3-8ms. **B**: monosynaptic reflex amplitude was quantified by calculating the absolute integral of the trace between 1.5 and 3ms, using 64 sweep-averages at 400µA. Median nerve stimulation results in a 15 fold greater response than radial nerve stimulation ($p<0.05$, $n=5$; Student's t-test). **C**: absolute integral values between 3 and 7ms show no significant difference between polysynaptic reflex amplitude elicited in the ulnar nerve by stimulation of the median or radial nerves ($p>0.05$, $n=9$; Student's t-test). Data are shown as mean ± standard error of the mean (SEM).

increase in the number of spikes evoked in the ulnar nerve (Fig. 2.3A). This phenomenon is known as wind-up. The number of spikes evoked in the ulnar nerve by the first stimulus at C-fibre intensity (4mA, 1ms) is generally modest but the bursts of activity intensify between the second and tenth stimuli (Fig. 2.3B) before reaching a gradually decaying plateau until the end of the stimulating period, when responses drop off rapidly. Although the precise number of spikes varied somewhat between individuals, the pattern of progressively increasing response to low frequency, repeated stimuli was extremely consistent. After the end of the 25 stimuli a period of heightened activity in the ulnar nerve in the absence of any stimulus is observed (Fig. 2.3C); this after-discharge would gradually subside back to resting levels of activity over 2-3 minutes. Radial nerve stimulation did not elicit wind up to the same extent. Rather, the number of spikes was mildly elevated throughout the stimulating period and dropped to basal levels immediately thereafter (Fig. 2.3D). Fig. 2.3E illustrates the difference between the number of spikes evoked by median and radial stimulation. Spikes per second of the recording period are shown, where 25 stimuli are delivered at 0.5Hz between the 20^{th} and 70^{th} seconds (Fig. 2.3E; 36-52s $p<0.001$, 32-62s $p<0.01$, 30-68s $p<0.05$).

Different methods of quantifying wind up responses have been described. In accordance with others (Castro et al., 2011) an area under the curve analysis was used, taking into account the 'input' (size of response to the first stimulus), which produces a measure of excess spikes. In addition, normalisation according to the baseline noise levels was performed to account for variations in whole nerve recording conditions. Figures attained from such calculations are referred to as wind-up and these are shown in Table 2.1. This analysis revealed a consistent difference in the number of spikes evoked between the median and the radial nerves (Fig. 2.3F; $p<0.05$). During the final 15 median nerve stimuli ulnar nerves exhibited a degree of 'wind-down', from the peak,

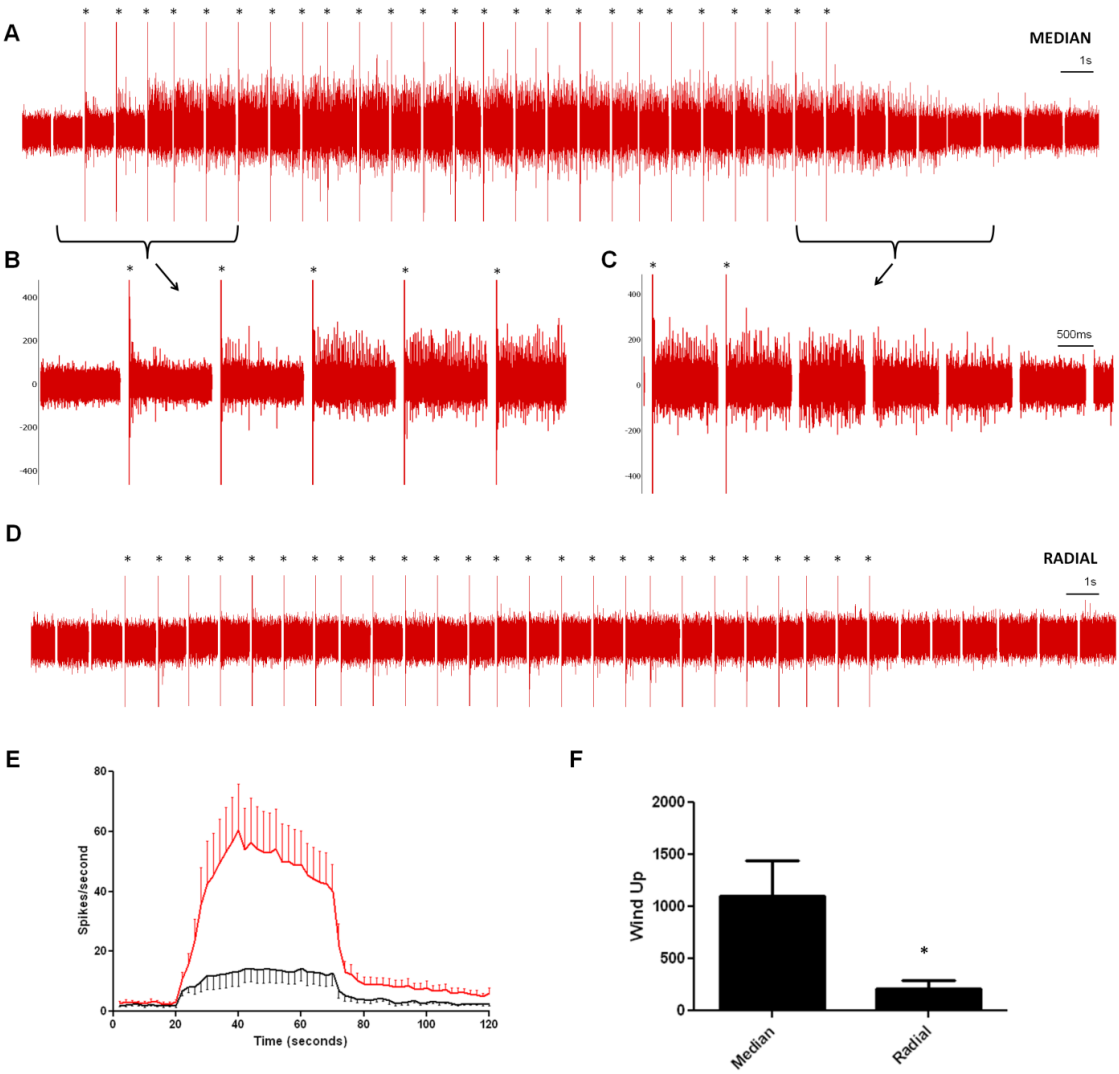

A
MEDIAN
1s
B
C
500ms
D
RADIAL
1s
E
Spikes/second
Time (seconds)
F
Wind Up
Median
Radial

Figure 2.3: Characterisation of high threshold reflexes. A: shown is an example of a whole ulnar nerve recording before, during and after stimulation of the median nerve at C-fibre intensity (4mA amplitude, 1ms square wave pulse) and low frequency (0.5Hz). 25 stimuli were delivered during each trial, indicated by asterisks. Evoked spikes increase during the stimulating period. **B**: enlarged view of ulnar nerve activity during the first five stimuli of a wind-up trial and for one second preceding the first stimulus. The number of spikes evoked by each successive stimulus increases progressively. **C**: wind-up trials are followed by a period of continuing elevated activity in the absence of stimuli for some seconds. Shown are the final two stimuli of a wind-up trial and a period of after-discharge. **D**: response of the radial nerve to a wind-up trial. Wind-up is not observed to the same extent as after median nerve stimulation. **E**: spike frequency plot quantifying the features of wind-up exemplified in **A-D**. The ulnar nerve has low levels of basal activity. Upon initiation of a wind-up trial (20 seconds), stimulating the median nerve, a rapid rising slope of increasing evoked activity occurs reaching a plateau after 10 stimuli (40 seconds). This level of activity slightly decays over the remainder of the trial and dramatically when stimulation ceases (70 seconds). Activity is elevated above basal levels for the remainder of the recording period (70-120 seconds). Radial nerve stimulation induces an increase in activity during the wind-up trial to a significantly lesser extent than during median nerve stimulation (36-52s $p<0.001$, 32-62s $p<0.01$, 30-68s $p<0.05$, $n=9$; two way RM-ANOVA) and ulnar nerve activity returns to basal immediately after cessation of stimulation. **F**: area under the curve quantification confirms significantly greater wind-up elicited by median vs. radial nerve stimulation ($p<0.05$, $n=9$; paired t-test). Data are shown as mean SEM.

usually around the 10th stimulus. By the end of the stimulating period the number of spikes elicited by the 25th stimulus was reduced by 33.0±12.2% from the peak levels of activity.

These results indicate that wind-up can be readily elicited in a flexor nerve by high intensity stimulation of a synergistic flexor nerve, but not by identical stimulation of an extensor nerve in the same plexus. This robust difference in capacity to produce wind-up in the ulnar nerve provides a convenient paradigm to study plasticity of spinal reflexes in the context of peripheral nerve injury.

2.3.2 *Effect of peripheral nerve injury on spinal reflexes*

Animals underwent electrophysiological testing approximately 12 weeks after receiving peripheral nerve injury, with either correct or crossover repair. The effect of injury was investigated using the paradigms for studying spinal reflexes described above.

Fast reflexes

Correct repair had no impact on the size of the monosynaptic reflex with the size of the short latency response almost identical to uninjured animals (Fig. 2.4A, black bars). Incorrectly repaired median nerves had fast latency waves of much greater variability but with a mean area similar to control animals. As we did not observe any fast latency response upon radial nerve stimulation in any nerve injury paradigm this was not measured.

The longer latency, low threshold polysynaptic reflexes in correctly repaired nerves were, again, unchanged for both median and radial nerve stimulation, compared to controls (Fig. 2.4B, C, black bars; $p>0.05$). Rats with incorrectly repaired nerves displayed a trend towards decreased polysynaptic reflex amplitude in the radial but not the median nerve (Fig. 2.4B, C, black bars; effect of injury $p=0.36$ for median and

	Injury	Monosynaptic (mV.s)		Polysynaptic (mV.s)		Wind-up (no. spikes)	
		Median (flexor)	**Radial (extensor)**	**Median (flexor)**	**Radial (extensor)**	**Median (flexor)**	**Radial (extensor)**
Untreated	Uninjured	19.0±4.0	1.3±0.05	63.7±10.8	50.3±8.4	1105±334	207±79
	Correct	18.9±5.7	n/a	65.9±13	40.0±9.9	247±102	51±20
	Crossed	17.1±10.0	n/a	44.3±9.3	14.5±3.6	264±67	363±239
ChABC-treated	Uninjured	19.3±6.9	n/a	71.8±17	51.6±13.1	911±241	241±85
	Correct	21.9±9.0	n/a	123.0±32.0	70.9±19.1	1007±133	526±246
	Crossed	29.1±1.0	n/a	110.0±20.2	46.5±11.9	701±197	825±231

Table 2.1: Values for reflex magnitude in electrophysiology experiments. Reflex amplitude in shown for low threshold mono- and polysynaptic reflexes. For wind-up the number of spikes, corrected for input and basal activity, is shown. Data are shown as mean ± SEM.

p=0.089 for radial). These results indicate that peripheral nerve injury has a minimal effect on the amplitude of median nerve monosynaptic and low threshold polysynaptic spinal reflexes, but which included a trend towards a decrease in crossed radial nerve low threshold polysynaptic reflex amplitude.

Wind-Up

Ulnar nerve wind-up in response to flexor stimulation was considerably reduced after both types of peripheral nerve injury (Fig. 2.6G, black bars; $p<0.01$). These results indicate that wind-up collapses after peripheral nerve injury and that the severity of the injury does not affect the size of the reduction: an approximately 80% reduction in flexor wind-up is observed after both correct and crossed repair of the median nerve.

Flexor motoneuron responses to extensor stimulation, which even in intact nerves are small, were not significantly altered after peripheral nerve injury (Fig. 2.6H, black bars; $p>0.05$). In all cases radial nerve wind-up does not significantly change, especially in the case of the correctly repaired nerve, but there is an interesting trend for misdirected nerves (where regenerated fibres now inappropriately innervate 'flexor' tissues) to exhibit a greater degree of wind-up. The mean wind-up in this group is sevenfold greater than the correct repair group, although the results were highly variable.

These results show a divergent effect of peripheral nerve injury on high and low threshold spinal reflexes. Whereas low threshold reflexes remain largely unchanged, high intensity stimulation uncovers a difference in wind up between injured and uninjured experimental groups implying some central reorganisation following nerve injury.

2.3.3 *Effect of ChABC treatment*

Effect of LV-ChABC on spinal reflexes after injury – fast reflexes

The size of responses elicited from the uninjured ulnar nerve after median nerve stimulation showed animals that received LV-ChABC had a monosynaptic compound action potential of the same size as untreated animals (Fig. 2.4A). Following nerve injury those animals with correctly repaired median nerves similarly evoked a monosynaptic wave of statistically unchanged amplitude after treatment (Fig. 2.4A) and animals with crossed median nerves showed a non-significant trend towards and increased amplitude with ChABC treatment (Fig. 2.4A).

The low threshold, polysynaptic reflexes for both the median and radial nerves were again unchanged when the intact nerves of animals treated with LV-ChABC were stimulated (Fig. 2.4B, C). However, in the presence of a peripheral nerve injury the size of responses recorded between 3-8ms were significantly increased for the median nerve after both correct repair and crossed repair (Fig. 2.4B; significant effect of treatment $p<0.01$). Radial nerve stimulation provoked an increased response in LV-ChABC treated animals with correctly repaired nerves and a response of pre-injury magnitude upon stimulation of crossed nerves (Fig 2.4C; effect of treatment $p=0.052$).

Overall, these results indicate that ChABC treatment does not affect the amplitude of the median nerve monosynaptic reflex, whether or not the animals have undergone peripheral nerve injury. However, low threshold polysynaptic reflexes from both nerves undergo changes in amplitude in the presence of ChABC, but only after nerve injury.

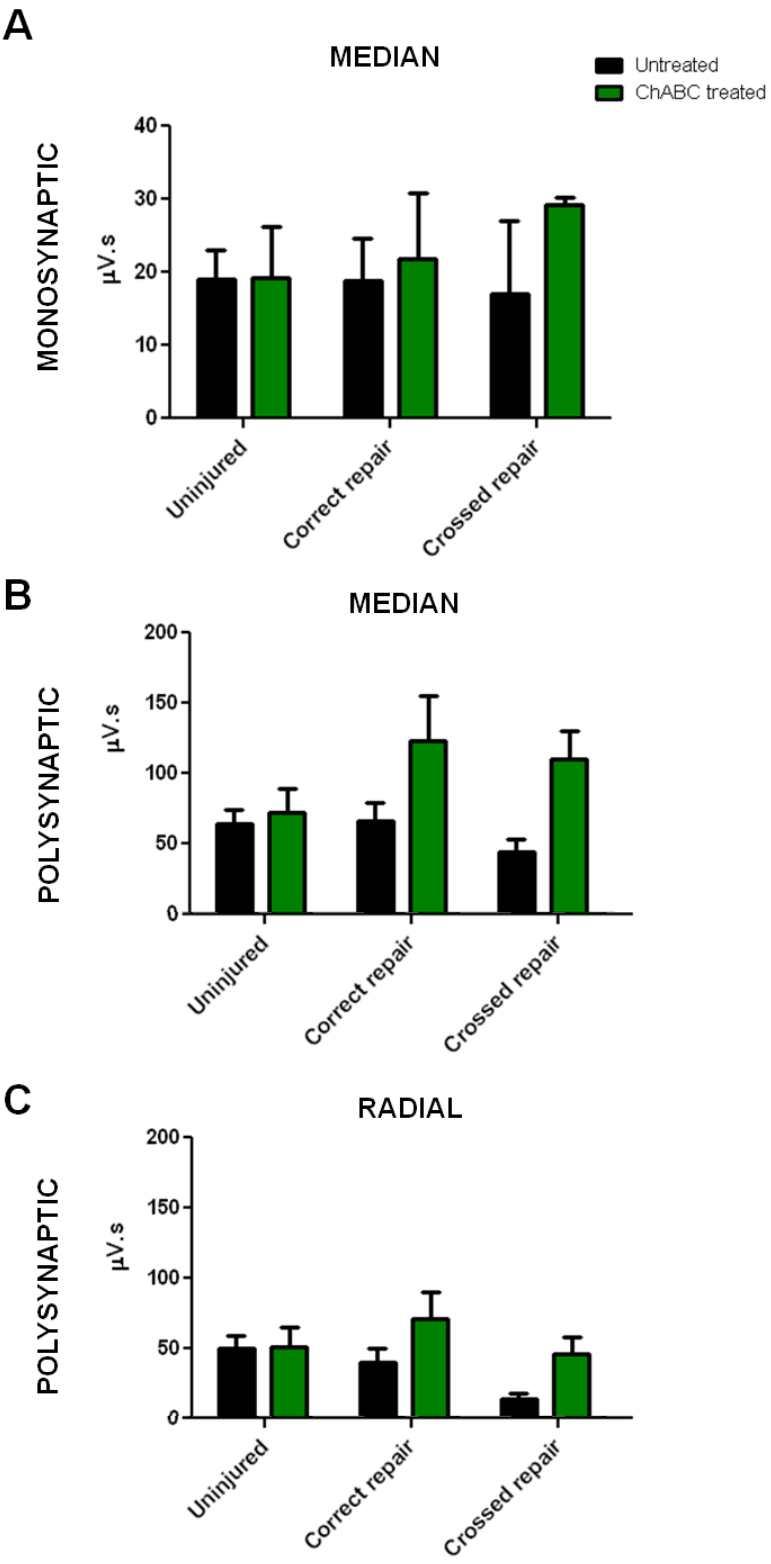

A
MEDIAN
Untreated
ChABC treated
MONOSYNAPTIC
µV.s
40
30
20
10
0
Uninjured
Correct repair
Crossed repair
B
MEDIAN
POLYSYNAPTIC
µV.s
200
150
100
50
0
Uninjured
Correct repair
Crossed repair
C
RADIAL
POLYSYNAPTIC
µV.s
200
150
100
50
0
Uninjured
Correct repair
Crossed repair

Figure 2.4: ChABC treatment enhances polysynaptic but not monosynaptic low threshold reflex amplitude after peripheral nerve injury. A: the amplitude of median nerve reflexes at monosynaptic latency (1.5-3ms) does not change in the presence of ChABC, regardless of whether or not the nerve has been injured (effect of treatment $p=0>0.05$; treated: n=6 uninjured, n=3 correct repair, n=3 crossed repair; two way ANOVA). **B**, **C**: low threshold polysynaptic reflexes produced upon both median (**B**) and radial (**C**) stimulation increase in amplitude in the presence of ChABC, but only after nerve injury (effect of treatment: $p<0.01$ for median, $p=0.052$ for radial; untreated: n=9 uninjured, n=7 correct repair, n=5 crossed repair; treated: n=8 uninjured, n=4 correct repair, n=6 crossed repair; two way ANOVA). Data are shown as mean ± SEM.

Effect of LV-ChABC on spinal reflexes after injury – wind-up

Both types of peripheral nerve injury studied caused a reduction in wind-up elicited by median nerve stimulation (Fig. 2.5A-C). Rats that received LV-ChABC in addition to peripheral nerve injury had a marked increase in response to C-fibre stimulation (Fig. 2.5E, F). Qualitatively, recordings from ChABC treated animals (Fig. 2.5D-F) appear to show many more spikes compared to spikes elicited from untreated nerve-injured rats (Fig. 2.5B, C). Changes after nerve injury with or without ChABC treatment are illustrated in spike frequency plots (Fig. 2.6). Here the collapse in median nerve wind-up after nerve injury (Fig. 2.6A, C, E) and its restoration (Fig. 2.6D, F) can be clearly observed. After nerve injury there is no change in the radial nerve activity of untreated rats after correct repair (Fig. 2.6C) but there is a slight increase after crossed repair, which occurred to variable degrees between individual animals (Fig. 2.6E). In contrast, radial nerve stimulation begins to elicit sizable wind-up responses in the nerve injury groups of animals treated with LV-ChABC, where none was present without treatment (Fig. 2.6D, F). Quantification of these results confirms the observed changes in spike frequency. Following administration of LV-ChABC median nerve wind-up was restored to pre-injury levels for rats that underwent correct repair; those that received crossed repair also showed a significant increase in wind-up, albeit to a lesser extent than the milder injury (Fig. 2.6G; significant effect of treatment $p<0.05$ in both cases). Intact animals treated with LV-ChABC have similar levels of wind-up to naïve rats. Radial nerve wind-up does not change following LV-ChABC administration to intact animals (Fig. 2.5D, 2.6H), but starts to appear after both types of nerve injury (Fig. 2.6D, F; significant effect of treatment $p<0.05$). The wind-down phenomenon that can be observed in control animals is still present in animals that received ChABC but no injury, albeit to a slightly lesser extent. The number of spikes after the 25^{th} stimulus was 67.0±12.2% of that after the 10^{th} stimulus for untreated and 85.3±6.4% for treated

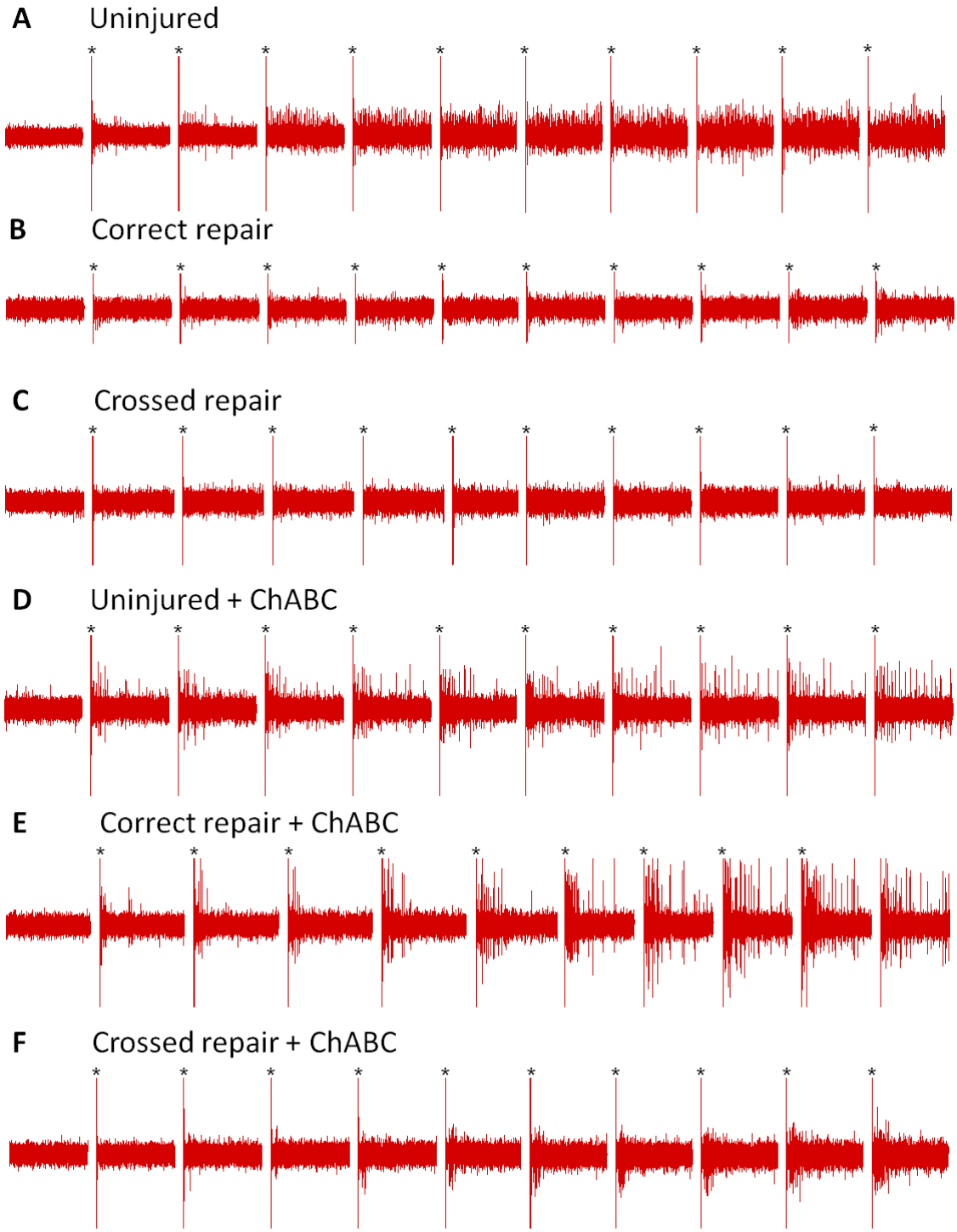

A Uninjured
B Correct repair
C Crossed repair
D Uninjured + ChABC
E Correct repair + ChABC
F Crossed repair + ChABC

Figure 2.5: Representative traces of wind-up trials illustrate differences between injury and treatment groups. Shown are the first 10 stimuli, indicated by asterisks. **A**: uninjured animals exhibit classic wind-up. **B**, **C**: wind-up is greatly reduced after peripheral nerve injury. **D**-**F**: ChABC treatment restores wind-up in uninjured and correctly repaired groups and increases wind-up in animals with crossed repair. The size of the evoked spikes also appeared to be enlarged in some animals that had been treated with ChABC (**D**, **E**).

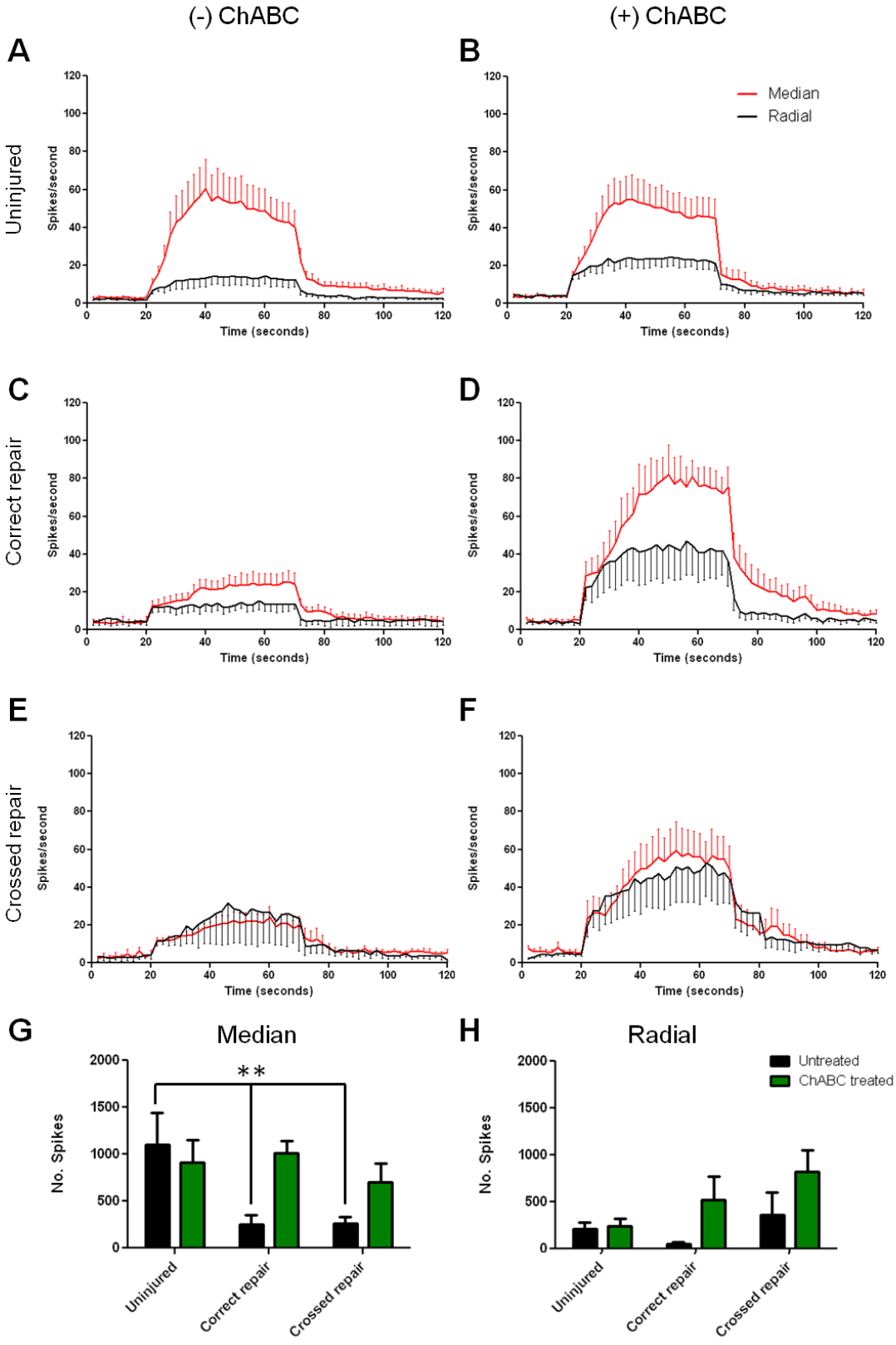

(-) ChABC
(+) ChABC
A
B
C
D
E
F
G
H
Uninjured
Correct repair
Crossed repair
Spikes/second
Time (seconds)
Median
Radial
Median
Radial
No. Spikes
**
Untreated
ChABC treated
Uninjured
Correct repair
Crossed repair

Figure 2.6: ChABC treatment induces plasticity of wind-up after peripheral nerve injury. A-**F**: spike frequency plots showing the number of spikes recorded per second before (0-20 seconds), during (20-70 seconds) and after (70-120 seconds) a wind-up trial, consisting of 25 stimuli at supramaximal C-fibre threshold (amplitude: 4mA, pulse width: 1ms). Wind-up evoked by median nerve stimulation (red) is robust in control animals (**A**) but reduced dramatically after peripheral nerve injuries of varying severity (**C**, **E**). In the presence of ChABC control animals exhibit unchanged levels of wind-up (**B**), while injured animals show significantly increased levels wind-up (**D**, **F**). Radial nerve wind-up (black) is modest under control conditions (**A**), remains so after correct repair (**C)** and increases somewhat after crossed repair (**E**). In the presence of ChABC radial nerve wind-up responses become larger (**D**, **F**). **G**: area under the curve quantification shows that ChABC does not change median nerve stimulation evoked wind-up magnitude in the absence of nerve injury but induces significant increases in amplitude when animals have been subjected to peripheral nerve injury. **H**: radial nerve stimulation evoked wind-up remains modest in uninjured animals in the presence of ChABC, but significantly increases in magnitude after both types of nerve injury. Both median and radial nerves had a significant overall effect of treatment, $p<0.001$, two-way ANOVA, but there were no significant differences between individual groups; untreated: n=9 uninjured, n=7 correct repair, n=5 crossed repair; treated: n=8 control, n=4 correct repair, n=6 crossed repair; two way ANOVA. Data are shown as mean ± SEM.

uninjured animals (Fig. 2.6A, B). In contrast, after injury ChABC treated animals maintain the plateau in the number of spikes that is reached at the peak of wind up. After the 25th stimulus the number of spikes was 110±11.1% of the number of spikes after the 10th stimulus for correct repair and 97.3±8.2% for crossed repair (Fig. 2.6D, F). This loss of wind-down suggests an increase in spinal excitability after peripheral nerve injury and ChABC treatment.

Taken together, the changes we observe in wind-up following ChABC treatment, namely restoration of median nerve wind-up and the appearance of novel wind-up in the radial nerve, as well as the loss of wind-down, are strong indicators of compensatory plastic changes occurring in the spinal cord as a result of CSPG digestion, but only apparent in nerve-injured animals.

Effect of LV-ChABC treatment on reaching behaviour

Since inaccurate reinnervation of sensorimotor targets after nerve injury and repair leads to poor recovery of fine motor control skilled paw movements were tested using the staircase test (Montoya et al., 1991). Rats with correctly repaired nerves recovered the ability to reach for sugar pellets over time, almost returning to baseline performance over the testing period of 14 weeks post-injury, reaching a plateau at 10 weeks (Fig. 2.7A; 30.75±0.25 pellets at baseline and 3.0±1.3 pellets at week 1 to 24.8±6.9 pellets by week 10). LV-ChABC treatment did not enhance the rate or degree of recovery, indeed the animals in this group exhibited a tendency towards performing less well than untreated animals on this task (Fig. 2.7A; untreated vs. ChABC, $p>0.05$). Animals in the group that received incorrect repair did not regain the ability to reach for and grasp more than one or two sugar pellets with their injured paw (Fig. 2.7B; 31.5±1.5 pellets at baseline and 4.5±2.5 pellets at week 1 to a maximum of 9.0±1.0 pellets by week 10).

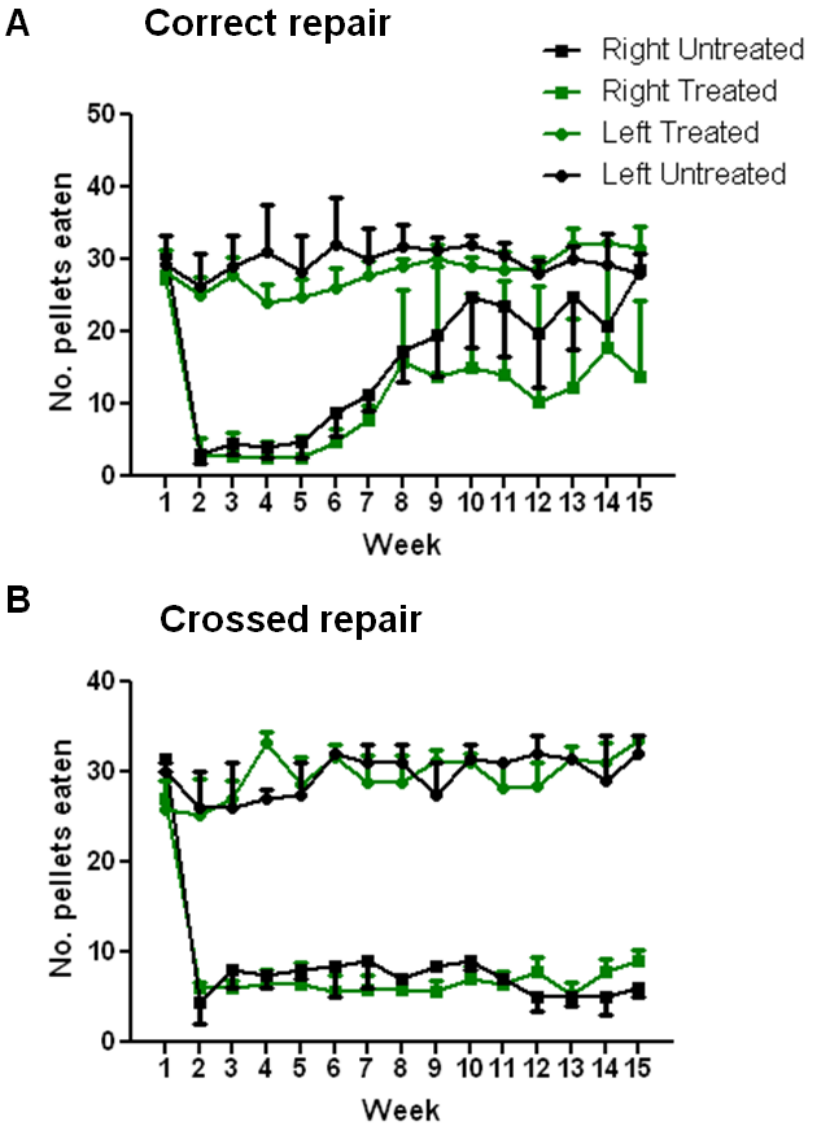


Figure 2.7: ChABC treatment did not improve reaching behaviour on the staircase test after peripheral nerve injury. A: animals with correctly repaired nerves regain the ability to reach for sugar pellets by 10 weeks post-injury. ChABC treated animals do not recover any better than untreated animals ($p>0.05$; n=4 untreated, n=5 treated; two way RM-ANOVA). **B**: animals with crossover nerve repair do not regain the ability to retrieve sugar pellets from the staircase, either with or without ChABC treatment ($p>0.05$; n=4 untreated, n=4 treated; two way RM-ANOVA). Data are shown as mean ± SEM.

LV-ChABC treatment did not improve the reaching ability of the rats in this injury group, although there was a trend towards improved scores in the last two weeks of testing (Fig. 2.7B; 9±1.3 vs. 6±1.0 pellets at 15 weeks, untreated vs. ChABC).

2.3.4 *Confirmation of CSPG digestion*

To confirm the presence of digested CSPGs in the spinal cord, the expression of chondroitin-4-sulphate (C-4-S) was measured. C-4-S is the protein stub region that is exposed after GAG chain cleavage from CSPGs by ChABC. We employed a gene delivery method to administer ChABC to the spinal cord via lentiviral vector. First, LV-ChABC was injected into the cervical spinal cord of naïve rats to investigate the extent of digestion of CSPGs. After a single injection of LV-ChABC sustained, widespread digestion of CSPGs was observed. This was visualised by the intense C-4-S immunostaining that is apparent 2 weeks after injection (Fig. 2.8A) and is maintained at the same level of intensity at 4 weeks (Fig. 2.8B) and up to 4 months after injection (Fig. 2.8C, D). Thus, this delivery method effectively degrades CSPGs in the spinal cord for the duration of the study period. To investigate the structure of the perineuronal net spinal cords from nerve injured, untreated and treated animals were examined for WFA reactivity at the end-point of the experiment (approximately 12 weeks after injury). WFA was observed surrounding the cell bodies of most spinal neurons in untreated, nerve-injured animals (Fig. 2.8F), but was not present around the neuronal cell bodies in the spinal cord of LV-ChABC treated rats (Fig. 2.8G). This shows that the perineuronal net is disrupted for many weeks after LV-ChABC injection.

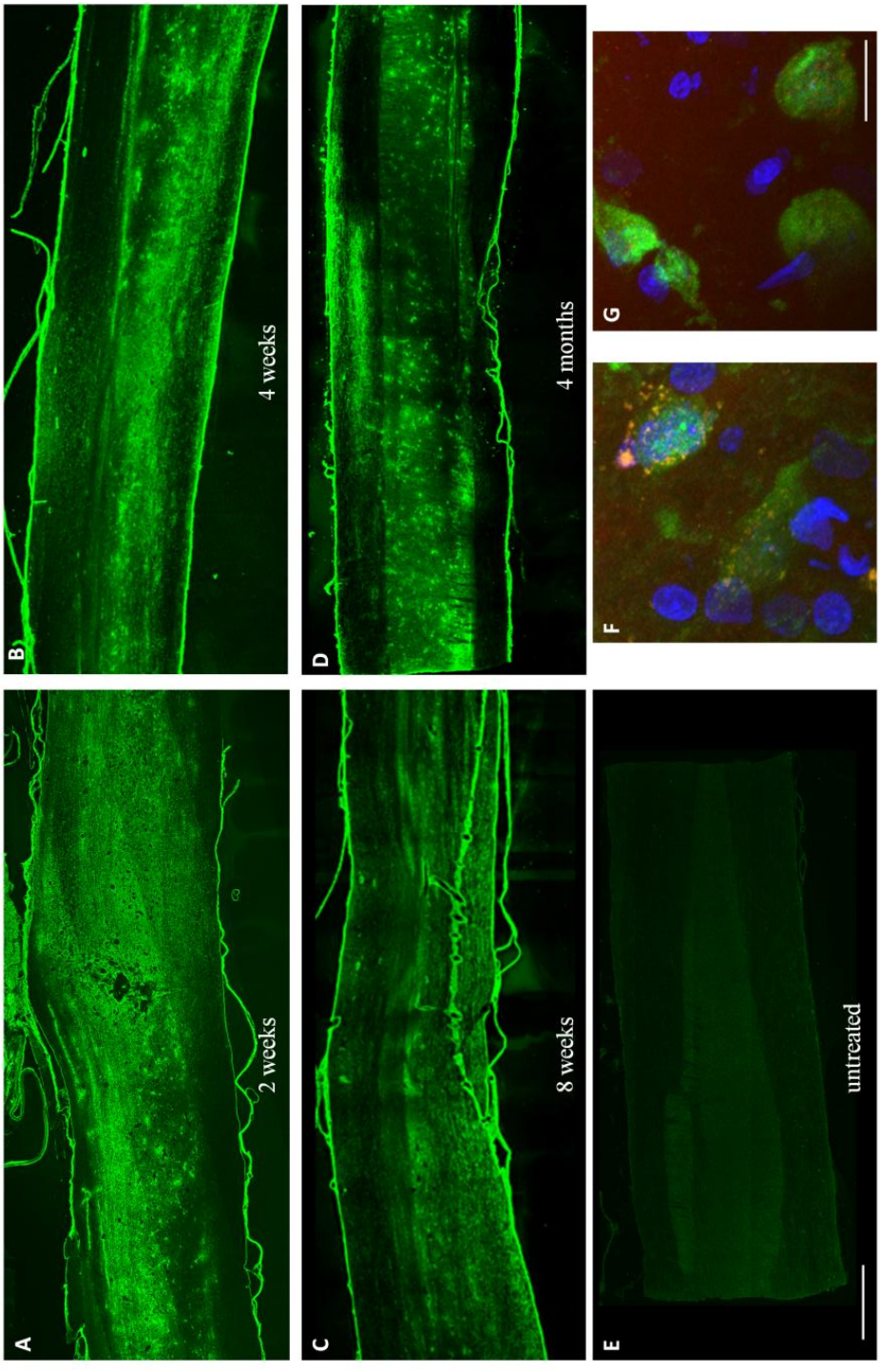

A
2 weeks
B
4 weeks
C
8 weeks
D
4 months
E
untreated
F
G

Figure 2.8: Lentiviral delivery of ChABC induces long-lasting CSPG digestion and perineuronal net disruption. A-D, LV-ChABC enzyme injection (1µl) into the intact spinal cord produces intense, widespread immounoreactivity to chondroitin-4-sulphate (C-4-S) epitopes from 2 weeks post-injection (**A**). Intensity and spread of digestion is maintained at 4 weeks (**B**), 8 weeks (**C**) and 4 months (**D**) after LV-ChABC injection. WFA reactivity indicates perineuronal nets surrounding some spinal neurons (**F**), which is no longer visible 12 weeks after injury (**G**). In (**A-D**) the tyramide amplification technique has produced some nonspecific staining on the edge of the spinal cord. Scale bars = 1mm (**A-E**), 20µm (**F, G**).

2.3.5 *Effect of LV-ChABC treatment on pain behaviour*

It is conceivable that a period of accentuated plasticity could lead to changes in connectivity and the formation of aberrant connections, potentially resulting in the development of spontaneous pain. Although it is known that repeated intrathecal injections of ChABC enzyme do not lead to pain (Barritt et al., 2006), the effect of sustained and widespread CSPG digestion has not been extensively studied. Therefore, withdrawal thresholds to mechanical and thermal stimulation were tested for animals that had undergone correct repair with or without LV-ChABC treatment. In addition, pain behaviour after formalin injection to the affected forepaw was assessed. Baseline testing revealed no significant difference in mechanical (15.1±0.9g vs. 16.7±0.8g, untreated vs. ChABC; $p>0.05$) or thermal (7.4±0.5s vs. 6.2±0.5s, untreated vs. ChABC; $p>0.05$) thresholds after LV-ChABC treatment. Over 10 weeks of post-injury testing, no sensitivity to non-noxious mechanical stimuli (Fig. 2.9A; $p>0.05$) or noxious thermal stimuli (Fig. 2.9B; $p>0.05$) developed. Similarly, pain behaviour after formalin injection into the forepaw exhibited the classic biphasic response of an intense early response during the first five minutes and a moderate later response from 15 to 45 minutes (Dubuisson and Dennis, 1977; Wheeler-Aceto et al., 1990). This did not differ between treated and untreated rats (Fig 2.9C; $p>0.05$). These results show that there is no increase in pain sensation with long-term CSPG digestion by ChABC in rats with nerve injury. The data also show there is no obvious sedative effect or motor impairment with this widespread and long-lasting digestion of spinal CSPGs.

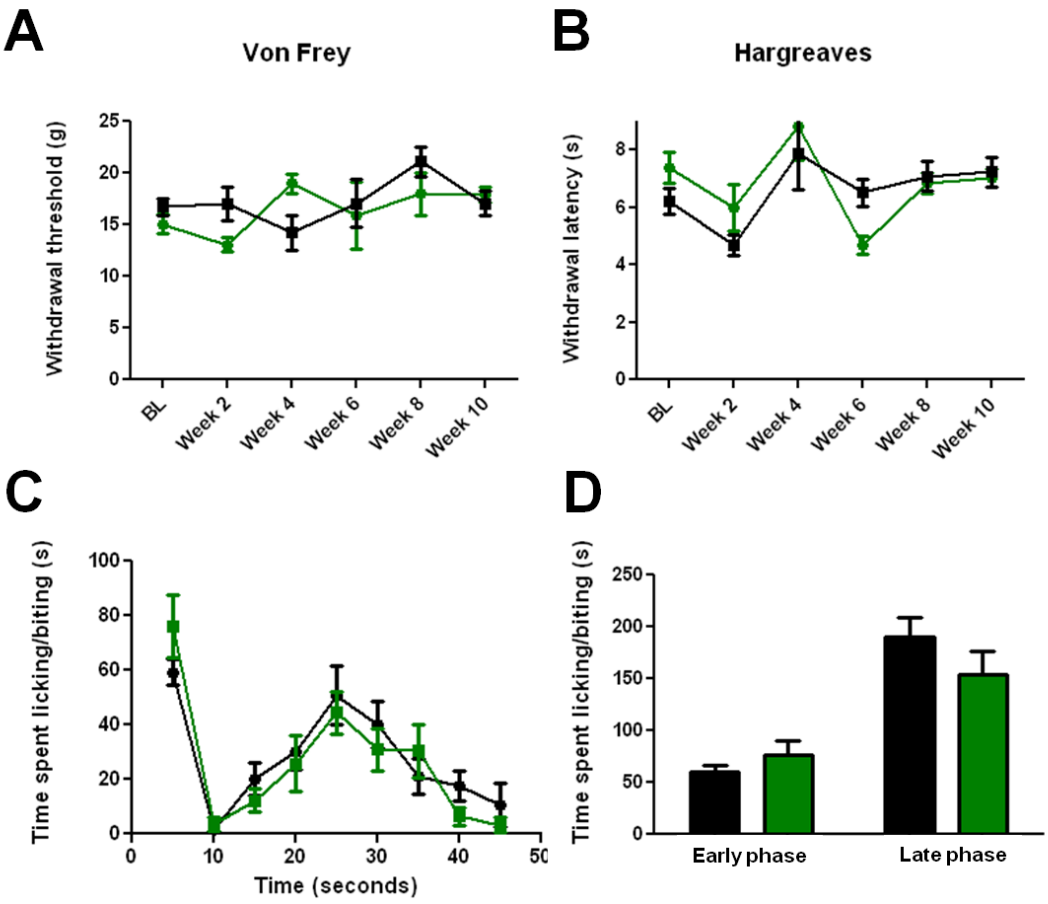


Figure 2.9: Long term ChABC treatment does not induce increased pain sensitivity following nerve injury and correct repair. ChABC treatment does not induce increased sensitivity to non-noxious mechanical (**A**; $p>0.05$, n=3 untreated, n=6 treated; two way RM-ANOVA) or noxious thermal stimuli (**B**; $p=0.67$, n=3 untreated, n=6 treated; two way RM-ANOVA). **C**: formalin-induced sensitivity is not enhanced in those animals that have received ChABC as well as nerve injury ($p>0.05$, n=3 untreated, n=6 treated; two way RM-ANOVA). **D**: no significant difference between groups was observed during the early and late phases of the response ($p>0.05$, n=3 untreated, n=6 treated; two way ANOVA). Data are shown as mean ± SEM.

2.4 Discussion

The hypothesis that CSPGs restrict the capacity for reorganisation of spinal connections following injury is supported by several of the findings from this investigation. Firstly, the amplitude of a low threshold, polysynaptic reflex could be increased after peripheral nerve injury, but only following treatment with ChABC, which degrades CSPGs. Secondly, wind-up of the ulnar nerve in response to median nerve stimulation, which collapses after nerve injury, could be protected by ChABC treatment after correct repair, or significantly increased after misrouting of the median nerve. Thirdly, wind-up of the radial nerve, which is minimal in the uninjured state, becomes significantly stronger in rats that have undergone both nerve injury and ChABC treatment. These results provide strong evidence that, by removing the inhibitory effects of CSPGs within the spinal cord, reorganisation of spinal circuitry after peripheral nerve injury is possible.

2.4.1 *Alteration of central connectivity*

Monosynaptic stretch reflex

Here, the well-studied monosynaptic stretch reflex was used to investigate spinal connectivity after nerve injury. We took advantage of the different responses of the ulnar nerve, a flexor, to flexor (median) or extensor (radial) nerve stimulation and explored the effect of injury on this reflex. Three months after injury, correctly repaired nerves showed a monosynaptic reflex of unchanged amplitude upon electrical stimulation. Recordings from motoneurons that have been axotomised reveal diminished synaptic input (Eccles et al., 1958; Kuno and Llinas, 1970) due to the loss of connectivity between Ia afferents and motoneurons (Mendell et al., 1974, 1976). However, if a cut muscle nerve successfully reinnervates muscle tissue, reflexes recover their normal properties (Carpenter et al., 1963; Farel, 1978), including the

functional connectivity between Ia afferents and motoneurons (Mendell and Scott, 1975) and our results are in agreement with these findings. It is likely that central monosynaptic connections with motoneurons are retained by Ia afferents despite potentially failing to reinnervate spindle receptors. It is also important to note that we were investigating heteronymous connections by stimulating damaged afferents and recording from intact motoneurons in the ulnar nerve, thus the finding of unchanged reflex amplitude is not surprising.

Incorrectly repaired nerves also had unchanged monosynaptic reflex amplitude, despite inaccurate peripheral regeneration (as confirmed by the observation of muscle twitch following electrical stimulation of crossed nerves proximal to the repair site). This was expected as it has been shown that regenerated Ia afferents do not need to innervate spindle receptors – regeneration into skin is adequate for functional connectivity with motoneurons to be maintained (Mendell et al., 1995). In this case, flexor axons that have been redirected to innervate extensor targets continue to connect equally strongly with flexor motoneurons, indicating an inappropriate monosynaptic connection between antagonistic muscles. Spinal ChABC treatment alone did not change the amplitude of the monosynaptic reflex and no change was observed after either type of injury, indicating that this reflex is a hard-wired, stereotypical response that is not susceptible to the plasticity-promoting properties of ChABC.

Fast polysynaptic reflexes

In contrast to the monosynaptic reflex studied above, low threshold stimulation of both flexors and extensors elicited polysynaptic responses in the ulnar nerve and the size of these reflexes were not significantly affected by injury. Following injuries of either severity, ChABC induced an increase in amplitude of these reflexes to approximately

double the size of those found in untreated animals, after both median and radial nerve stimulation. In the case of the median nerve, these reflexes were also substantially greater than those evoked in the ulnar nerves of naive animals. Interestingly, ChABC treatment did not change the size of polysynaptic reflexes of animals without nerve injuries. It therefore appears that nerve injury alone is not sufficient to cause a change in this spinal reflex but that, in an environment of depleted CSPGs, changes in connectivity become apparent. In addition, this finding shows that existing connections are not non-specifically altered by ChABC in the absence of nerve injury.

Wind-up

Wind-up is a well-known phenomenon that is a form of short-term plasticity that can be demonstrated by stimulating a peripheral nerve repetitively, at C fibre strength. First described by recording from dorsal horn neurons (Mendell and Wall, 1965), the use of whole nerve motoneuron recordings has also been described (Schouenborg and Sjolund, 1983; Woolf and Wall, 1986). Stimulating and recording in the periphery is advantageous as the outcome measure is a physiological measure of an integrated, modulated spinal reflex (Herrero et al., 2000). This is important because plastic changes may be mediated at different levels and by various neuronal cell types. In agreement with others, recordings showed that repetitive stimuli at supramaximal C fibre strength produced a progressively increasing discharge that peaked after around 10 stimuli (Schouenborg and Sjolund, 1983; Gozariu et al., 1997; Solano and Herrero, 1999), before reaching a gradually diminishing plateau until the end of the 25 stimuli. After the end of the stimulating period, spike discharge remained elevated for some time but returned to baseline levels of activity within minutes. These features were consistent between animals, despite the inherently variable nature of the absolute number of spikes elicited by a single wind-up trial (Castro et al., 2011). Median nerve stimulation

robustly induced wind-up recorded in the ulnar nerve, whereas radial nerve stimulation using identical parameters produced a much more modest response. This illustrates the difference in response of a flexor nerve to synergist compared with antagonist nerve stimulation. The difference between the responses of the two nerves provided a paradigm within which to investigate plasticity.

Both types of nerve repair resulted in significantly depressed levels of wind-up at a chronic time point. Collapse of wind-up occurred irrespective of the severity of misdirection of axons, suggesting that this phenomenon is due to central rather than peripheral consequences of nerve injury and regeneration. This is supported by the previous morphological observations that synaptic vesicles are lost from peptide-containing primary afferent terminals of axotomised neurons (Zhang et al., 1995). It is believed that a synergy between glutamate and peptidergic signalling is a feature of wind-up (Randic et al., 1990; Thompson et al., 1993), hence this depletion could explain the failure of wind-up upon electrical stimulation. In contrast to the results of this Chapter, others have found that wind-up of normal magnitude can be elicited by electrical stimulation in animals that have undergone ligation or transection of the sciatic nerve (Laird and Bennett, 1993; Xu et al., 1995; Chapman et al., 1998; Jergova et al., 2012). However, these other studies were all studying hindlimb nerves, whereas the present observations apply to forelimb nerves. In addition, two of the above studies were on animals that exhibited increased pain sensation (Laird and Bennett, 1993; Chapman et al., 1998), for which no evidence was found here. In addition, this was the first description of wind-up in the context of the repaired nerve, as opposed to ligation or axotomy without repair.

Median nerve stimulation in animals that had undergone correct repair and ChABC treatment produced wind up that was similar to that of naïve rats. Indeed, spike frequency plots showed that the pattern of wind up appeared even more excitable than that of a naive animal. The number of stimuli delivered before activation and the amount of after-discharge were both elevated, while the plateau reached stayed horizontal rather than decaying. These observations indicate a restoration of connectivity within the spinal cord and suggest a state of heightened central excitability. The incorrectly repaired median nerve also showed significantly increased wind-up after treatment with ChABC and a loss of wind-down. However this was not as robust as for the correctly repaired median nerve. Interestingly, radial nerve stimulation after ChABC treatment caused the appearance of novel wind-up in the ulnar nerve, in those rats that had undergone nerve injury. This indicates the formation of new or more effective polysynaptic connections between injury-affected radial nerve afferents and ulnar nerve efferents. Cafferty et al. (2008b) showed that ChABC delivered to the intact spinal cord in a spared root rhizotomy paradigm resulted in receptive fields that were larger than those of intact controls, whereas a restoration of normal function would have resulted in a restitution of normal sized receptive fields. Median nerve wind-up results similarly appear to show "recovery" of magnitude of response. However, in the light of the appearance of novel activity in the radial nerve and in the low threshold polysynaptic reflexes described above, it is more likely that a new, alternative function has emerged. This is supported by the emergence of new characteristics of median nerve wind-up, such as the loss of wind-down.

No change in wind-up was found when the uninjured nerves of ChABC-treated animals were tested. In fact, wind-up after either median or radial nerve stimulation in these animals was statistically undistinguishable from intact controls. This is consistent with

the changes observed in low threshold polysynaptic reflexes described above. These results suggest that, when the inhibitory influences of CSPGs are mitigated, the capacity for anatomical reorganisation in response to nerve injury becomes realised, as has been proposed previously (Cafferty et al., 2008b). As both injury paradigms in both nerves result in increased polysynaptic reflex activity, it could be argued that this is a change in recording conditions between groups or non-specific hyperexcitability due to, for example, lower anaesthetic level. If this were the case, one would expect the size of all reflexes to be increased but this is not the case; as discussed above monosynaptic reflex size does not change between treated and untreated animals and is therefore acting as an internal control, indicative of consistent recording conditions between animals.

Wind-up is a response to noxious inputs but it occurs in normal animals and its presence does not necessarily signal a pain state. In this Chapter, wind-up was studied as a measure of short-term plasticity. It is present in normal animals and collapses after nerve injury. The protection against this collapse by ChABC treatment suggests the restoration of some capacity for plasticity. However, it is not clear whether this is adaptive or maladaptive for the animal. Behavioural testing for thermal hyperalgesia, mechanical allodynia and inflammatory pain did not detect any maladaptive changes.

For both high and low threshold reflexes, no evidence emerged to suggest that ChABC treatment promoted respecification of connections so they became appropriate to the tissues innervated after regeneration. That is, flexor nerves that were misdirected to innervate extensor territories did not behave exactly as would be expected of an extensor and vice versa, rather the plastic changes observed were more general in

nature. Possible mechanisms for the observed ChABC mediated changes are discussed below.

2.4.2 *Forelimb function*

To investigate whether spinal ChABC could lead to an improvement in skilled forelimb movements, such as reaching and grasping, we tested rats weekly on the staircase test (Montoya et al., 1991). Rats with correctly repaired nerves recovered reaching ability over time but ChABC treatment did not enhance this process, indeed treated animals tended to perform worse on this task. This finding is inconsistent with a previous study where intraspinal ChABC and a similar nerve injury paradigm were employed and animals with correct repair showed an improvement in functional recovery of grasping (Galtrey et al., 2007). There is an important difference between the nerve injuries used in the present study and that by Galtrey et al. (2007), in their case the authors manipulated the median and ulnar nerves, while here the median and radial nerves were injured. This is significant because both the median and ulnar nerves are flexors while the radial nerve is an extensor. The deficit caused by the injuries in the present study will therefore affect both flexor and extensor forelimb functions, and the increased severity of the injury may explain the discrepancy between the behavioural results. In addition, behavioural data for the correctly repaired nerve injury group includes results for animals that, having failed to retrieve pellets for a number of trials following injury, gave up attempting retrieval, even though physically they may have been able to complete the task. However, in agreement with Galtrey et al. (2007), rats with misdirected axon regeneration fail to recover any reaching ability in the present study, with or without ChABC treatment.

As suggested by the electrophysiological findings, countering the restrictive influence

of perineuronal nets may lead to an increase in general connectivity (low threshold, polysynaptic) and effectiveness of connections (wind-up), perhaps indicating the potential for functional improvement. Others have shown a weak effect of ChABC alone on behavioural improvement but a strong synergistic effect of ChABC and rehabilitative training (Garcia-Alias et al., 2009). This observation led the authors to suggest that ChABC mediates the formation of multiple connections and rehabilitative training selects appropriate connections and eliminates inappropriate ones. Therefore, it may be important to combine ChABC-activated plasticity with rehabilitation, as did the study by Galtrey et al. (2007), in order to promote recovery of a skilled forelimb function such as paw reaching, which is not a behaviour commonly exhibited by animals in their home cages (Garcia-Alias et al., 2009).

The question of whether ChABC can improve behavioural outcome is not conclusively addressed by the results of the present study. A less demanding behavioural test, such as the grip strength or horizontal ladder test, which detects more subtle improvements, may be a more promising avenue for future investigation. In addition, experimental groups used for this experiment were small, ideally larger groups would be utilised for such a complex task with varying levels of engagement.

2.4.3 *Mechanisms of ChABC mediated plastic changes*

ChABC has been reported to mediate its effects on functional reorganisation through (i) anatomical changes in connectivity as a result of sprouting, (ii) enhancing axonal conduction, (iii) neuroprotective effects, and (iv) alteration of synaptic efficacy. Any of these mechanisms could contribute to the changes seen here. The phenomenon of sprouting of intraspinal axons after ChABC treatment is commonly described (Barritt et al., 2006; Massey et al., 2006; Garcia-Alias et al., 2009; Soleman et al., 2012). It has

been reported that MAP1B immunoreactivity, indicating newly grown processes, is increased in the ChABC-treated spinal cord after periperhal nerve injury (Galtrey et al., 2007). This sprouting is likely to be due to a tipping of the balance towards axonal growth, which, as it has recently been shown, can be regulated by activity of the protein tyrosine phosphatase σ receptor (Coles et al., 2011), for which CSPGs are ligands (Shen et al., 2009). As we observed evidence of not only recovered but enhanced synaptic transmission it is plausible that the formation of novel connections by afferents on postsynaptic targets has occurred. It has recently been shown that CSPGs, when applied to the spinal cord, can depress axonal conduction and that ChABC can prevent this decline (Hunanyan et al., 2010). These findings suggest that CSPGs are inhibitory to conduction. Since conduction failure in some central branches of primary sensory axons has been described (Titmus and Faber, 1990), CSPG digestion might allow more widespread invasion of presynaptic axonal arbours, thereby increasing post-synaptic responses. Additionally, perineuronal nets, of which CSPGs are a major component, have been implicated in the stabilisation of synapses (Hockfield et al., 1990) and disruption of perineuronal net components can effect short and long-term synaptic changes (Bukalo et al., 2001; Frischknecht et al., 2009; Dityatev et al., 2010b; Morellini et al., 2010). Here we report enhanced spinal reflex activity, which could reflect the strengthening of existing spinal connections, consistent with the involvement of CSPGs in regulating synaptic efficacy. It has also been suggested that latent, silent synapses exist (Wall, 1995) and that the unmasking of these weaker connections, induced by the retraction of competing connecting terminals from injured afferents, may be responsible for the observed increase in reflex activity (Cafferty et al., 2008b), especially in the light of the enhanced axonal conduction described above. Another proposed mechanism for the increased reflex activity observed is that ChABC has a neuroprotective function. Damaged sensory neurons show widespread changes in structure and function and

ameliorating such effects could allow for more effective electrophysiological signalling. ChABC has been shown to prevent the atrophy of layer V cortical neurons following axotomy at the levels of the spinal dorsal columns (Carter et al., 2008). After peripheral nerve injury neurotransmitter levels decrease and second order neurons undergo changes, so neuroprotection, perhaps mediated by the release of trophic factors from the extracellular matrix after exposure to ChABC, may be responsible for the observed increase in neural activity. In the absence of injury neurotransmitters/neuromodulators are maintained at normal levels, so this may explain the observed injury-specific changes effected by ChABC. It is intriguing that we found evidence for reorganisation only with respect to polysynaptic and not monosynaptic reflexes. This is likely to reflect the greater susceptibility of these reflexes to modulation due to an increased number of loci for such effects.

2.4.4 *Conclusion*

This study has shown clear evidence for ChABC mediated plasticity of spinal reflexes after peripheral nerve injury. While most studies focus on CSPGs in the context of the glial scar, we have demonstrated their importance in the intact spinal cord. Reorganisation of intact CNS circuitry following nerve injury in the periphery presents a window of opportunity to promote functional recovery.

CHAPTER 3

Gene delivery of Chondroitinase ABC promotes functional repair after spinal cord injury

3.1 Introduction

3.1.1 *Contusion Injury*

Spinal cord injury (SCI) can lead to devastating permanent deficits in motor, sensory and autonomic function. It is a major clinical problem, as it has been estimated that at least 2.5 million people worldwide live with a SCI and that over 132,000 people sustain a SCI every year (http://www.campaignforcure.org). By examining the histology of human traumatic SCI, the types of injury can be classified into four groups: solid cord injury, contusion, massive compression and laceration (Bunge et al., 1993). The most frequent human SCI is contusion injury, which is a blunt trauma leading to bruising of the spinal cord. The injury features intact meninges, a fluid-filled cavity or cyst and damage to grey and white matter (Norenberg et al., 2004). It is appropriate to use rats to study contusive SCI as they have been found to bear functional and morphological similarities to human SCI (Metz et al., 2000; Scheff et al., 2003). Rodent models of contusion injury have advanced since the weight drop method originally described by Allen (1911) and now feature the use of sophisticated pneumatic-controlled devices that allow precise regulation of the severity of the injury, resulting in predictable functional deficits (Scheff et al., 2003). In addition, detailed information regarding biomechanics may be monitored, such as the velocity or the degree of displacement (Onifer et al., 2007). Moderate rat contusion injury, such as is used in the present study, has recently been extensively characterised by James et al. (2011), who showed that axonal conduction in long distance sensory projections is severely impaired after spinal contusion. They also show a limited, stereotypical degree of spontaneous functional recovery as demonstrated by behavioural and electrophysiological techniques. Thus, this clinically relevant, well-characterised injury is an ideal model to study functional recovery following SCI and experimental treatments aimed at promoting repair.

3.1.2 *Chondroitin sulphate proteoglycans in the injured spinal cord*

Following SCI, a reactive process takes place involving the accumulation of glial cells which surround the wound to form a glial scar (Reier et al., 1983; Fawcett and Asher, 1999). In an injury where the meninges are not breached, such as that used in the present study, the glial scar is mainly composed of reactive astrocytes (Silver and Miller, 2004) that re-establish the blood-brain barrier and seal the wound (Faulkner et al., 2004; Sofroniew, 2009). Despite its advantages, the glial scar also represents a barrier to regenerating axons, which is both physical and chemical in nature. A major inhibitory component of the scar is the chondroitin sulphate proteoglycan (CSPG) family of molecules, which are inhibitory to neurite outgrowth *in vitro* (McKeon et al., 1991; Brittis et al., 1992; Dou and Levine, 1994; Friedlander et al., 1994; McKeon et al., 1995). CSPGs are upregulated after spinal cord injury in rodent models, with the highest level of expression at 8-14 days and levels remaining heightened into chronic stages (Jones et al., 2003b; Tang et al., 2003; Iaci et al., 2007). Although injured spinal cord neurons attempt regeneration after transection (Kerschensteiner et al., 2005), the inhibitory CSPG-rich milieu of the lesion site precludes significant regeneration. In addition, CSPGs are known to block conduction following spinal cord injury (Hunanyan et al., 2010), thus hindering the function of any spared axons.

3.1.3 *Chondroitinase ABC after spinal cord injury*

Clear evidence for the inhibitory role of CSPGs after spinal cord injury was provided by the finding that functional recovery after SCI can be enhanced by treatment with ChABC (Bradbury et al., 2002). Subsequently, many studies have shown the potential of ChABC to enhance regeneration of sensory axons (Yick et al., 2003; Cafferty et al., 2007; Shields et al., 2008) and motor axons (Iseda et al., 2008; Garcia-Alias et al.,

2009; Wang et al., 2011). In addition to increasing the regenerative ability of injured axons, it is increasingly evident that ChABC can induce anatomical reorganisation through collateral sprouting of intact or injured fibres. Sprouting has been observed following ChABC treatment of SCI in the rat (Barritt et al., 2006) and the cat (Tester and Howland, 2008; Tom et al., 2009). A functional improvement has often accompanied ChABC-induced sprouting (Massey et al., 2006; Cafferty et al., 2008b; Tester and Howland, 2008; Garcia-Alias et al., 2009).

Most studies have used partial transection models of spinal cord injury to study the effectiveness of ChABC treatment (Bradbury and Carter, 2011). However, it is not clear whether similar efficacy can be achieved in a more clinically relevant model of spinal injury. One study by Caggiano et al. (2005) used intrathecally delivered ChABC and showed some functional recovery, but only after a severe injury. In that study a clip compression model was used, which mimics the pathology of a contusion injury (Caggiano et al., 2005). In another study, this time in the context of cervical contusion injury, and when combined with a peripheral nerve graft, ChABC treatment could elicit functional improvement (Houle et al., 2006). Other studies have shown no beneficial effects after contusion injury (Tom et al., 2009; Jakeman et al., 2010). The effectiveness of ChABC at promoting recovery after injury may be linked to the duration of enzyme activity and the severity of the injury. Although administration of a single dose of ChABC has been shown to be beneficial after lesions of particular tracts, this does not appear to be the case in the context of contusion injury, perhaps due to the more severe nature of this type of injury. Most studies showing functional improvements have used a repeated administration regimen (Bradbury et al., 2002; Caggiano et al., 2005; Fouad et al., 2005).

3.1.4 *A genetic approach to targeting CSPGs*

After a single injection of ChABC enzyme, immunoreactivity for digested CSPGs persists for a considerable time (Bruckner et al., 1998), but enzyme activity appears to decrease rapidly 1-2 days post-injection (Lin et al., 2008). In order to lengthen the duration of enzymatic activity, genetic approaches to CSPG modification have emerged. These have included transgenic mice expressing ChABC under the GFAP promoter (Cafferty et al., 2007) and the administration of viral vectors expressing ChABC (Curinga et al., 2007; Jin et al., 2011). These studies have met with limited success, as bacterial proteins are not designed for secretion from mammalian cells and are susceptible to interference from the eukaryotic N-glycosylation system, preventing secretion of active enzyme. Modification of the ChABC enzyme through directed mutagenesis has allowed mammalian cells to express and secrete active enzyme (Muir et al., 2010). *In vivo* expression of this modified enzyme via lentiviral vector (LV) has been shown to promote sprouting of injured corticospinal axons in the rat spinal cord (Zhao et al., 2011). Thus, ChABC delivery via LV is an attractive strategy as it allows targeting of the enzyme to the desired area, expression of ChABC by the cells in the vicinity of the injury and longer lasting CSPG digestion than can be achieved by enzyme injection alone.

3.1.5 *Aims of the Chapter*

This chapter describes the use of genetically modified ChABC packaged into an LV (LV-ChABC) to treat adult rats with spinal cord injury. The model used is the clinically applicable contusion injury, applied at thoracic level. Behavioural and electrophysiological techniques were employed to investigate functional changes after injury and following treatment with ChABC. Results show that LV-ChABC treated

animals display both increased conduction across the lesion and plastic changes to spinal reflexes below the lesion, and that LV-ChABC treated animals perform better on a test of sensorimotor function. In addition, anatomical assessment showed remarkable improvements in injury pathology, notably by reducing cavity size and altering the appearance of the glial scar. We propose that long-lasting, widespread CSPG digestion allows considerable repair and functional improvement following traumatic spinal cord injury.

3.2 Methods

3.2.1 *Surgical procedures*

*Animals***.** 98 adult female Sprague Dawley rats (200-220g) were used in these studies. Behavioural testing was carried out on animals that underwent contusion injury only (n=15) and animals that underwent contusion plus either LV-ChABC (LV-3, n=15) or a control vector (LV-GFP, n=16). Subsets of these animals also underwent electrophysiology (contusion only, n=10; LV-GFP, n=10; LV-3, n=10) and pain testing (contusion only, n=7; LV-GFP, n=8; LV-3, n=7). An additional group were used only for electrophysiology (LV-1, n=4; LV-2, n=4; ChABC enzyme; n=5). CSPG digestion assessment and pain studies were carried out on a further group of naive animals (LV-1, n=6; LV-2, n=6; LV-3, n=13; ChABC, n=6; saline, n=8). All surgical procedures were performed in accordance with UK Home Office regulations (European Communities Council Directive of 24th November 1986 (86/609/EEC)).

Lentiviral vectors. The ChABC gene used is a modified version designed to optimise secretion from mammalian cells (Muir et al., 2010). The vectors used to deliver the modified ChABC gene were integrating, self-inactivating LVs, which have been described in detail elsewhere (Zhao et al., 2011). LV-1 and LV-2 expressed ChABC under control of the cytomegalovirus promoter and the LV-3 used the phosphoglycerate kinase promoter. All three vectors were pseudotyped with VSV-G and packaged using a second generation system, as described previously (Naldini et al., 1996a; Hendriks et al., 2007). Viral titres, obtained by a p24 antigen ELISA assay, were 99µg/ml (LV-1) and 479µg/ml (LV-3), corresponding to 6x10^5 TU/µl and 1x10^6 TU/µl, respectively. Viral titre for LV-2, obtained by Q-PCR, was 1.44x109gc/ml, corresponding to 8x10^{5}TU/µl.

*Intraspinal injections***.** For recovery surgery, sterile precautions were used. Rats were anaesthetised with 60mg/kg ketamine and 0.25mg/kg medetomidine, diluted in sterile saline and administered i.p. Body temperature was monitored rectally and used to regulate a homeothermic blanket. A single dose of 5mg/kg carprofen was given subcutaneously at the time of induction. Rats were prepared for surgery by shaving and disinfecting the dorsal surface of the back over the scapulae. After skin incision the layers of muscle were dissected to expose the lower cervical vertebrae. A partial laminectomy of the C5 vertebra was performed and gel foam soaked in 2% lignocaine was placed on the dura for two minutes, then a small incision was made in the dura using small spring scissors (Fine Science Tools) and lignocaine applied for a further two minutes. A single intraspinal injection was delivered into the left side of the cord, consisting of 1µl of LV-ChABC (LV-1, LV-2, or LV-3), saline or protease free ChABC enzyme (Seikagaku Corporation; 10U/ml). Injections were performed using a glass capillary pulled to a tip diameter of 20µm, connected to a 10µl syringe and driven by a microdrive pump (Harvard Apparatus) at 0.25µl/min. The capillary was left in place for 2 minutes after the injection. Overlying muscle and skin were sutured in layers. 1mg/kg atipamezole hydrochloride was administered subcutaneously to reverse the anaesthetic and animals recovered in an incubator. At 2 and 8 weeks post-injection n=3 animals from each group, except saline, were sacrificed in order to assess CSPG digestion.

*Spinal cord injury and treatment***.** Animals were anaesthetised and prepared for surgery as described above. Laminectomies were performed at vertebral level T10 and the vertebral column was stabilised using Adson forceps. An Infinite Horizon impactor (Precision Systems Instrumentation) was used to deliver a moderate severity (150kdyn)

contusion injury, through the intact dura mater, to the exposed spinal cord. Immediately after injury animals received intraspinal injections of ChABC, LV-ChABC or LV-GFP (0.5µl 1mm rostral and caudal to the injury site as above) and a control group received no injection. Overlying muscle and skin were sutured in layers and animals received atipamezole (1mg/kg), saline (3-5ml) and enrofloxacin (5mg/kg) subcutaneously. Rats were left to recover in cages on heated blankets overnight. Saline and enrofloxacin continued to be administered for 3 and 7 days postoperatively, respectively. Manual bladder expression was performed twice daily until reflexive emptying returned, typically 6-9 days after injury. Animals underwent behavioural testing for 10 weeks before electrophysiological assessment was carried out.

3.2.2 *In vivo electrophysiology*

*Conduction studies***.** Rats were terminally anaesthetised with an intraperitoneal injection of 1.25g/kg urethane (Sigma-Aldrich). The spinal cord was exposed from vertebral level T7 to L5, the dura mater was removed and exposed nervous tissue was covered with mineral oil. Silver ball stimulating electrodes were placed approximately 5mm rostral and caudal to the injury, over the midline. Tiny teased filaments from the left and right L3 to S1 dorsal roots were individually mounted on silver wire hook electrodes for the recording of impulses running antidromically after dorsal column stimulation. Single units, reflecting the activity of single nerve fibres, were recorded and quantified from each of these filaments while stimulating above or below the lesion site (Fig. 3.3A). First, the number of units present in each filament was counted while stimulating caudal to the lesion (typically 5-10 units per filament). Stimulation was then applied rostral to the lesion while again counting units in the same filament. In this way the number of nerve fibres capable of conducting through the lesion could be calculated.

The dorsal columns were stimulated using 0.2ms duration square wave pulses at a frequency of 1Hz and at incrementally increasing intensity (0-800μA). Measurements of interelectrode distance were made at the end of each experiment to allow calculation of conduction velocity, which was measured for each unit and then averaged for each animal.

*Wind-up***.** As described in detail in the previous chapter, peripheral nerve recordings can be used to study the phenomenon of wind-up of flexor motorneurons. In these experiments spinal reflexes below the level of the injury, between hindlimb nerves, were examined. Rats were terminally anaesthetised as above and the left sciatic nerve and its branches exposed. The motor nerve to biceps femoris and the sural nerve were dissected free from surrounding connective tissue and cut distally. The common peroneal nerve was dissected free but left intact. Skin flaps from the incision formed a pool which was filled with mineral oil. Whole nerve recordings were individually made from the motor nerve to biceps femoris and the common peroneal nerves, which were mounted on silver wire hook electrodes in turn. Electrical stimulation was applied to the sural nerve, also mounted on hook electrodes. A train of 25 supramaximal stimuli was delivered to the sural nerve at a stimulus intensity of 4mA (1ms square wave pulse at a frequency of 0.5Hz). Recordings of biceps femoris or common peroneal nerve activity for 1 second after each impulse were captured using LabChart software (AD Instruments). Nerve activity during the 20 seconds preceding and the 50 seconds following each wind up trial was also recorded. Three trials were carried out for each nerve, with an interval of 5 minutes between trials to allow the nerves to return to baseline levels of resting activity. For the biceps femoris nerve, multi-unit recording of all spikes 25% greater than the mean noise level was made, typically producing 5 spikes per second under resting conditions. The number of spikes that reached this

threshold was recorded for each second before, during and after the period of stimulation and plotted as a graph. For each animal, recording parameters were not changed between biceps femoris and common peroneal nerve recordings and it was noted that the common peroneal had higher resting levels of activity, typically 100 spikes per second. For this nerve the number of spikes elicited by stimulation in addition to those present at baseline levels of activity were captured. To quantify wind-up from spike frequency plots, area under the curve analysis was performed. This value was then normalised to baseline nerve activity levels and input (the number of spikes discharged during the second following the first stimulus). The normalisation serves to control for differences in recording conditions and nerve excitability and is calculated as follows:

$$\text{Wind-up} = (\text{total spikes}) - ((\text{input} \times 25) - (\text{basal} \times 25))$$

Total spikes = sum of spikes recorded during the stimulating period. Basal = average number of spikes per second in the 20 seconds prior to the stimulating period.

3.2.3 *Behavioural assessment*

Basso, Beattie and Bresnahan locomotor score. Open field hindlimb locomotor function was assessed using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). This involved placing the rat in a circular 1 metre diameter open field and assessing the movement of both hindlimbs during locomotion over a period of 4 minutes. Criteria include joint movements, paw placement, weight support, consistency of stepping, coordination, toe clearance and trunk stability. Each hindlimb is scored on a 22 point scale with a maximum score of 21; these are averaged to give

each animal's overall score. Testing was performed on days 2, 5 and 7 after injury and weekly thereafter for 10 weeks.

Horizontal ladder. Animals were trained daily for 1 week to run across a 1 metre long horizontal ladder with unevenly spaced rungs. On the final day of training a Sony DCR-SX30E Handycam was used to record each rat running three times across the ladder. Videos were analysed in slow motion and the total number of hindlimb foot slips were quantified to give each animal a baseline score. A score of 1 was assigned to a slip that resulted in the entire paw going below the level of the rung. Animals were tested weekly, beginning 7 days after injury, for a total of 10 weeks.

Sensory testing. As in the previous chapter, mechanical and thermal thresholds, as well as response to formalin injection, were assessed to investigate whether LV-ChABC has an effect on nociceptive processing. We tested forepaw pain sensitivity after cervical injection of LV-3 in uninjured animals. Mechanical withdrawal thresholds were assessed using a dynamic plantar anaesthesiometer (Ugo Basile). Animals were placed in clear plexiglass cubicles on top of a metal grid and allowed to acclimate for 15 minutes. A computer-controlled stimulus was applied to the forepaw that applied a linearly increasing force ramp. A cut-off of 50g was imposed to prevent any tissue damage. The force necessary to elicit paw withdrawal was recorded. Thermal hyperalgesia was assessed by applying a radiant heat source to the plantar surface of the forepaw and measuring the time taken for the rat to withdraw its paw (Hargreaves et al., 1988). The time taken to withdraw was measured three times for each paw and the mean latency was analysed. For both mechanical and thermal testing, the withdrawal threshold of each forepaw was calculated as the average of 3 consecutive tests with at least 5 minutes between each test. Measurements were taken before spinal injection on

three separate days for baselines, 3 days post-injection and weekly thereafter. Formalin testing was performed at the end of the study (12 weeks after injury) as previously described (Dubuisson and Dennis, 1977). Briefly, 50µl of 5% formalin was injected under the skin of the plantar surface of the right forepaw. The rat was then placed in a plexiglass observation chamber and the time spent licking or biting was measured over 45 minutes, in 5 minute bins.

3.2.4 *Tissue processing and histology*

Tissue preparation. Animals were deeply anaesthetised using sodium pentobarbitone (80mg/kg, i.p.) and transcardially perfused with 200ml heparinised saline followed by 400ml paraformaldehyde (4% in 0.1M phosphate buffer (PB)). Spinal cords were dissected and post-fixed in 4% paraformaldehyde for 2 hours at 4^{o}C, then transferred to 20% sucrose in 0.1M PB for 48 hours at 4^{o}C for cryoprotection. The tissue was blocked in OCT embedding compound and frozen for cryostat sectioning. 20µm-thick sagittal or transverse sections were cut and serially mounted onto glass slides.

Immunohistochemistry. Transverse sections were double stained for glial fibrillary acidic protein (GFAP; a marker for mature astrocytes) and NeuN (a marker for neuronal cell bodies). After incubation in 10% normal donkey serum (30 minutes), sections were incubated in PBS with 0.2% Triton and 0.1% sodium azide containing monoclonal mouse anti-NeuN (1:500; Millipore) and rabbit anti-GFAP (1:2000; Dako) overnight at room temperature. After three PBS washes, sections were incubated with donkey anti-rabbit Alexa 488 (1:1000; Invitrogen) and donkey anti-mouse Alexa 546 (1:1000; Invitrogen) for 2 hours at room temperature. The method for visualisation of CS-GAG digestion by virally expressed ChABC is described in detail in the previous

Chapter (2.2.4). Briefly, frozen sagittal sections were incubated in the following (with three PBS washes between each step): normal donkey serum (10%, 30 minutes), mouse anti-C4S (1:5000, overnight; ICN Biochemicals), horse anti-mouse biotinylated secondary antibody (1:400, 90 minutes; Jackson ImmunoResearch), ABC reagent (1:250, 20 minutes; Vector Labs), biotinyl tyramide (1:75, 10 minutes; PerkinElmer Life Sciences) and ExtraAvidin TRITC (1:200, 2 hours; Sigma). Slides were coverslipped with Vectashield mounting medium (Vector Laboratories). Images were taken on a Zeiss LSM 710 upright confocal microscope.

Tissue sparing analysis. Eriochrome cyanine R histochemistry was used to differentiate grey and white matter, as described previously (James et al., 2011). Sections were dehydrated in an ethanol series (5 minutes each), cleared in Histochoice (5 minutes), rehydrated in a reverse ethanol series followed by distilled water (5 minutes each) and then left in a solution containing 0.16% eriochrome cyanine R, 0.5% sulphuric acid and 0.4% iron chloride for 10 minutes to stain myelinated fibres. Sections were then washed twice in distilled water, differentiated in 0.5% ammonium hydroxide (30 seconds) and washed twice in distilled water. Finally, sections were dehydrated and cleared, as above, and mounted using DPX. A Zeiss AxioCam microscope camera was used to take pictures of sections at 600µm intervals throughout the lesion site. Images were analysed using AxioVision software. The perimeters of the spinal cord and of the cavity were traced and the total area for each calculated. Cavity area as a percentage of total cord area was quantified every 600µm, up to a distance of 3.6mm rostral and caudal to the lesion epicentre, which was defined as the section from each animal with the largest cavity area.

3.3 Results

3.3.1 *Comparison of ChABC-expressing vectors*

In initial studies, naïve animals received single intraspinal injections of one of three different LVs expressing ChABC into the cervical spinal cord and these were compared to single injections of ChABC enzyme. LV expression of ChABC was driven by either CMV (LV-1 and LV-2) or by PGK (LV-3) promoters. The extent of CSPG digestion after application of each LV or ChABC enzyme was assessed by measuring chondroitin-4-sulphate (C-4-S) expression in the spinal cord at 2 or 8 weeks after injection. C-4-S is the protein stub region that is exposed after ChABC-mediated GAG chain cleavage from CSPGs. ChABC enzyme produces a localised area of intense C-4-S immunoreactivity by 2 weeks post-injection (Fig. 3.1A) and this becomes less intense by 8 weeks (Fig. 3.1B). The three LVs produced very different patterns of digestion from each other. After a single injection of LV-1 or LV-3 sustained, widespread digestion of CSPGs was observed as visualised by intense C-4-S immunostaining that is present at 2 weeks (Fig. 3.1C, G). Over the course of 8 weeks, CSPG digestion caused by LV-1 becomes more widespread but less intense (Fig. 3.1D). LV-3 induced C-4-S expression remains widespread and becomes more intense (Fig. 3.1H). Application of both LV-1 and LV-3 led to widespread CSPG digestion, with C-4-S observed over several spinal segments. These findings contrasted with the digestion pattern observed after injection of LV-2, which was restricted to the tissue surrounding the injection site (Fig. 3.1E, F) and was not so intensely expressed. These results show that administration of LVs expressing ChABC can lead to efficient transduction of cells and expression of active enzyme, leading to effective degradation of spinal CSPGs. In the case of two of the LVs tested, the digestion achieved was long-lasting and widespread.

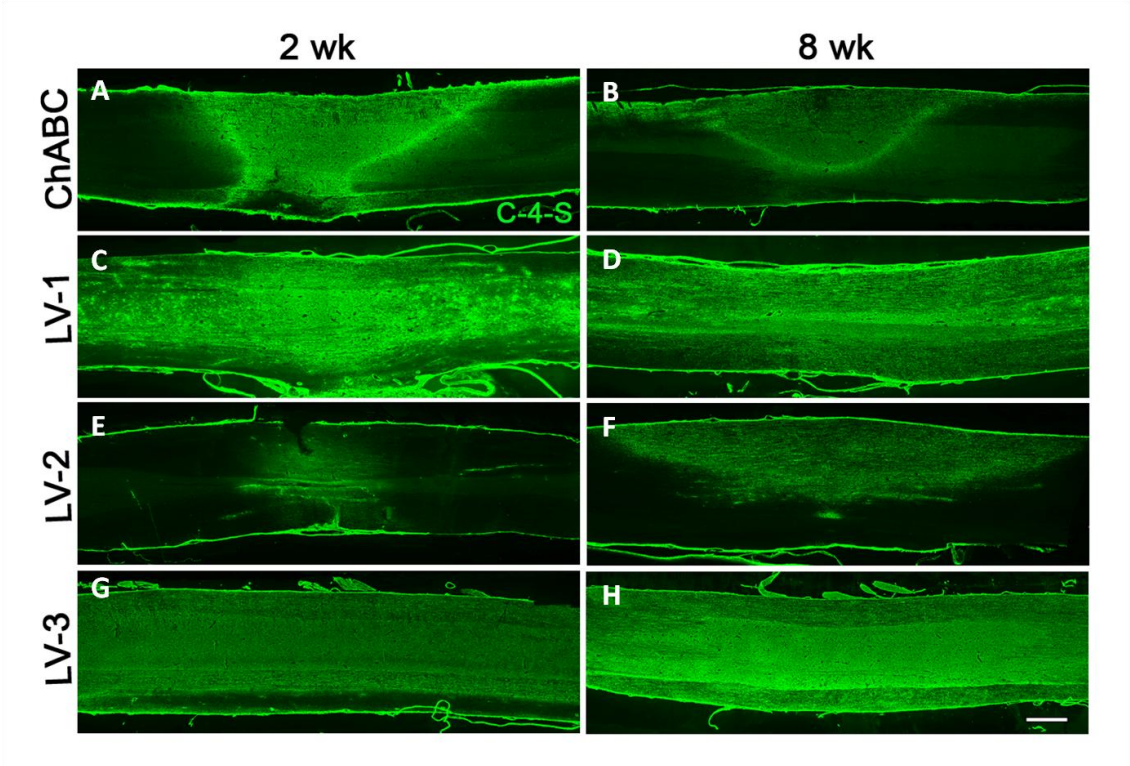

2 wk
8 wk
ChABC
LV-1
LV-2
LV-3
A
B
C
D
E
F
G
H
C-4-S

Figure 3.1: Lentiviral vector ChABC delivery leads to long-lasting and widespread CSPG digestion. Sagittal spinal cord sections were immunostained for digested CSPGs, shown by C-4-S staining (green). Naïve animals received a single injection of ChABC enzyme (**A**, **B**), LV-1 (**C**, **D**), LV-2 (**E**, **F**) or LV-3 (**G**, **H**) and spinal cords were stained for C-4-S at 2 (left) and 8 (right) weeks after injection. After ChABC enzyme injection a localised region of CSPG digestion is visible at 2 weeks (**A**) that becomes less intense by 8 weeks (**B**). LV-1 injection leads to intense C-4-S staining by 2 weeks (**C**), which becomes more widespread but less intense after 8 weeks (**D**). LV-2 injection leads to a small area of CSPG digestion by 2 weeks (**E**) and spreads a very short distance rostral and caudal from the injection site by 8 weeks (**F**). CSPG digestion is already very widespread 2 weeks after LV-3 injection (**G**) and remains so, with even more intense C-4-S staining by 8 weeks after injection. Scale bar = 1mm.

3.3.2 *Effect of long-lasting CSPG digestion on pain behaviour*

As mentioned in the previous chapter, it is possible that a prolonged period of accentuated plasticity could lead to the formation of inappropriate connections and the development of spontaneous pain. Of the three ChABC-expressing LVs, LV-2 and LV-3 led to the most CSPG digestion. In the previous chapter we found that the LV-2 vector did not lead to any heightened sensitivity after spinal injection into the uninjured spinal cord. Here we tested whether spinal injection of another LV that leads to extensive CSPG digestion, LV-3, similarly does not cause pain. We tested withdrawal thresholds to mechanical and thermal stimulation for uninjured animals that had received saline or LV-3 injection. Pain behaviour after formalin injection into the forepaw was also assessed. No significant differences were observed between LV-ChABC and saline animals in withdrawal responses to non-noxious mechanical stimuli or noxious thermal stimuli in the 12 weeks following injury (Fig. 3.2A, B; $p>0.05$ for both tests). The thresholds did decrease over time for both groups and in both tests, presumably due to rats learning to anticipate the probe over time. Formalin testing was carried out at the end of the experiment and exhibited the classic biphasic response of an intense early response during the first 5 minutes, followed by a lull in pain behaviour and the re-emergence of a later response between 15 and 45 minutes. This revealed no hypersensitivity in the animals that were treated with LV-ChABC versus the group that received saline, in either the late or early phase of the formalin response (Fig. 3.2C, D; $p>0.05$). These results show that there is no increase in pain sensation in naïve rats with long-term CSPG digestion.

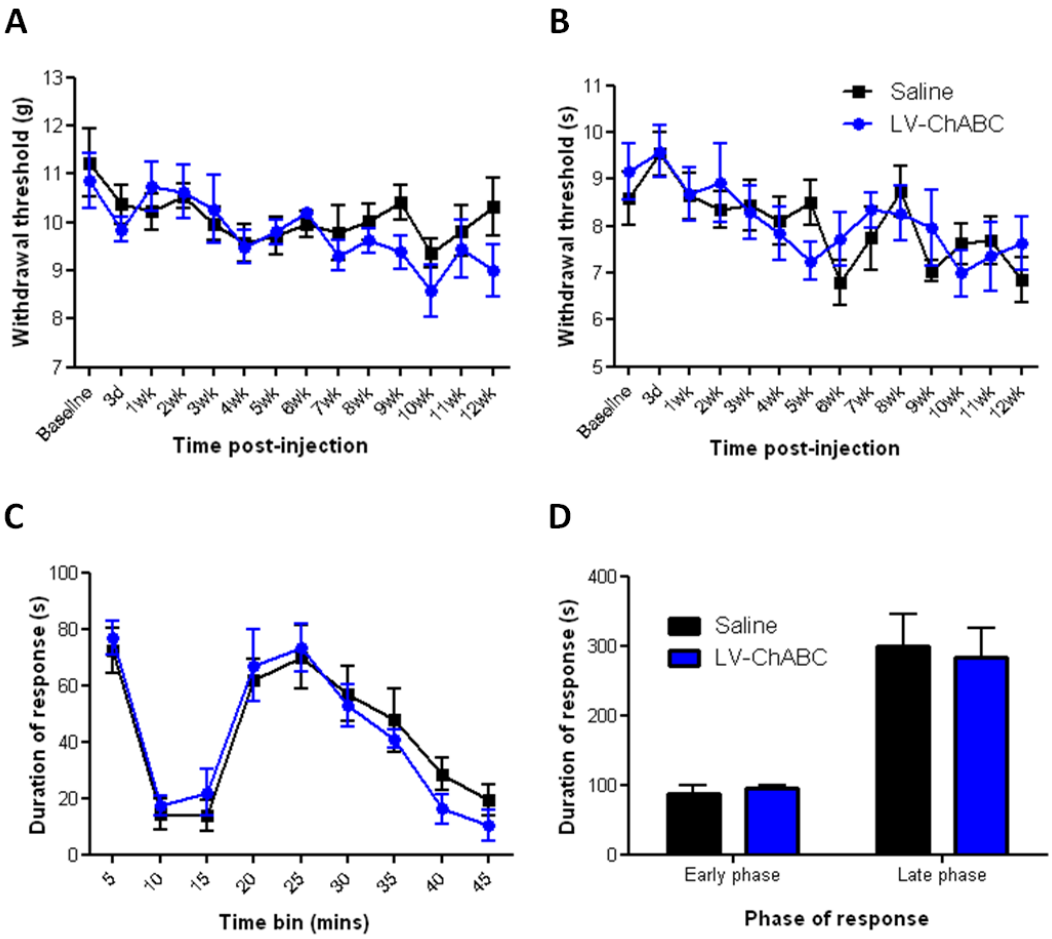
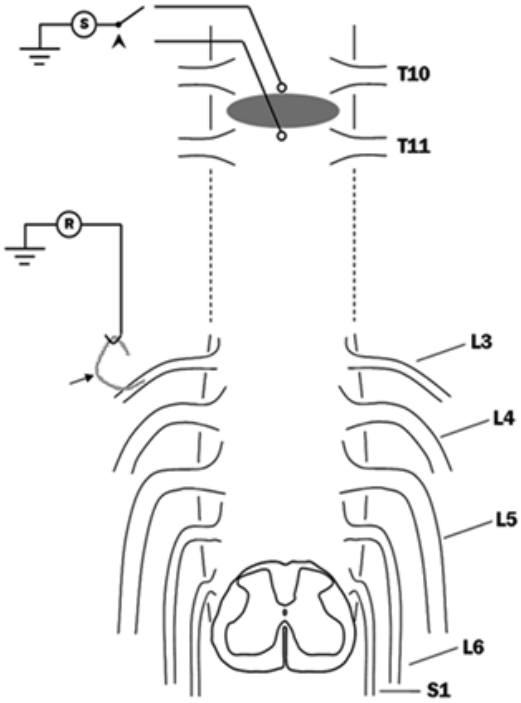

A
Withdrawal threshold (g)
13
12
11
10
9
8
7
Baseline
3d
1wk
2wk
3wk
4wk
5wk
6wk
7wk
8wk
9wk
10wk
11wk
12wk
Time post-injection
B
Withdrawal threshold (s)
11
10
9
8
7
6
5
Saline
LV-ChABC
Baseline
3d
1wk
2wk
3wk
4wk
5wk
6wk
7wk
8wk
9wk
10wk
11wk
12wk
Time post-injection
C
Duration of response (s)
100
80
60
40
20
0
5
10
15
20
25
30
35
40
45
Time bin (mins)
D
Duration of response (s)
400
300
200
100
0
Saline
LV-ChABC
Early phase
Late phase
Phase of response

Figure 3.2: Persistent and widespread CSPG digestion in the naïve spinal cord does not lead to pain hypersensitivity. LV-ChABC treatment does not induce increased sensitivity to non-noxious mechanical (**A**) or noxious thermal (**B**) stimuli ($p>0.05$ for both tests, n=7 LV-ChABC, n=8 saline; two-way RM ANOVA). **C**, formalin induced sensitivity is not enhanced in animals that have received LV-ChABC ($p>0.05$, n=7 LV-ChABC, n=8 saline; two-way RM ANOVA). **D**: no significant differences in pain behaviour were observed during the early and the late phases of the formalin response. ($p>0.05$, n=7 LV-ChABC, n=8 saline; two-way ANOVA). Data are shown as mean ± SEM.

3.3.3 *Comparison of efficacy of ChABC-expressing vectors on spinal conduction*

As CSPGs are reported to block axonal conduction after spinal injury (Hunanyan et al., 2010), we investigated whether administration of LV-ChABC could improve conduction after a moderate (150kdyn) contusion injury, 10 weeks after injury. The population of neurons studied in these experiments were sensory axons that have long-distance ascending axonal projections running in the dorsal columns. This tract was activated by stimulating below the lesion site and recording from single antidromically activated fibres running in teased dorsal root filaments, as shown in Fig 3.3A. The number of fibres in a particular filament was quantified and then stimulation of the same tract above the lesion was performed, allowing quantification of the number of fibres capable of conducting through the lesion. Spinal cord contusion led to a substantial reduction in the percentage of fibres able to conduct through the injury site, with only 10% of units elicited below the injury also present when stimulating above the lesion. Animals that were treated with LV-3 showed a significantly increased percentage of fibres able to conduct across the lesion (Fig. 3.3B; 24.7±4.3% following LV-3 compared with 10.2±0.8% following contusion only; $p<0.01$) and there was also a non-significant trend towards improved conduction after treatment with LV-1 (Fig. 3.3B; 16.7±4.5% following LV-1; $p>0.05$). In contrast, there was no improvement in conduction after administration of a control LV expressing GFP, ChABC enzyme or LV-2 (Fig. 3.3B; 12.5±2.1%, 9.6±0.9% and 7.6±1.5%, respectively; $p>0.05$). These results show that axonal conduction, which is severely impaired by spinal contusion injury, can be partially restored by mitigation of the inhibitory influence of CSPGs. The degree of restoration of function appears to correspond to the extent of digestion, since treatment with ChABC enzyme and LV-2, which both achieve only modest and localised CSPG digestion did not lead to an increase in conduction, while LV-3

A


S
R
T10
T11
L3
L4
L5
L6
S1

B

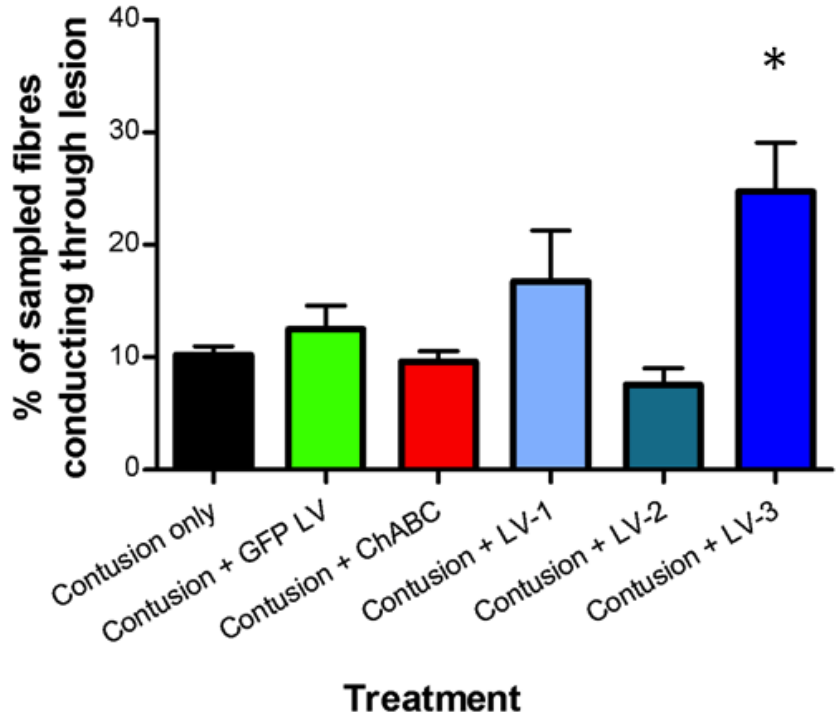
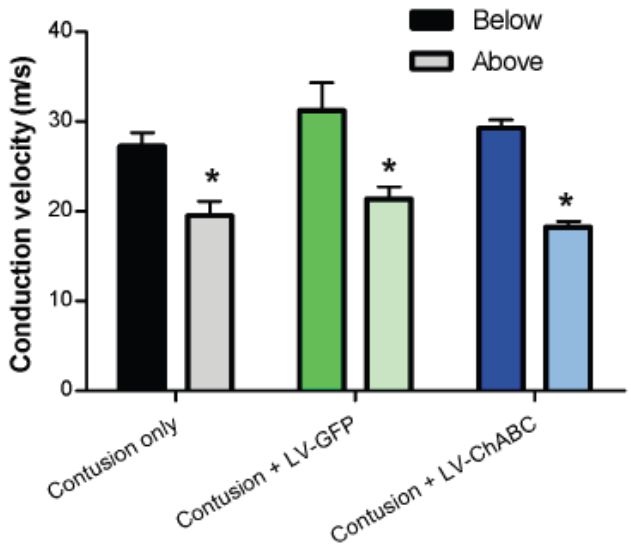

% of sampled fibres conducting through lesion
40
30
20
10
0
*
Contusion only
Contusion + GFP LV
Contusion + ChABC
Contusion + LV-1
Contusion + LV-2
Contusion + LV-3
Treatment

Figure 3.3: Changes in axonal conduction following contusion injury and LV-ChABC treatment. A: Diagram illustrating electrophysiology preparation. Stimuli are applied caudal to the lesion while antidromic impulses are recorded from tiny filaments teased from the L3 to S1 dorsal roots. Once the number of units and their latency in a given fibre has been established, a stimulus is then applied rostral to the lesion in order to establish the number of units that conduct through the lesion. **B**: 10 weeks following contusion injury alone the number of sampled fibres able to conduct through the lesion is low (~10%, n=5). Administration of LV-GFP, ChABC enzyme or LV-2 does not lead to any increase in conduction ($p>0.05$, n=4 LV-2, n=5 ChABC and LV-GFP; two-way ANOVA). Treatment with LV-1 leads to a non-significant increase in the number of conducting fibres ($p>0.05$, n=4, one-way ANOVA) and treatment with LV-3 led to a significant increase in the percentage of fibres able to conduct across the lesion (*=$p<0.01$, n=5; one-way ANOVA).

A


Below
Above
Conduction velocity (m/s)
40
30
20
10
0
*
*
*
Contusion only
Contusion + LV-GFP
Contusion + LV-ChABC

B

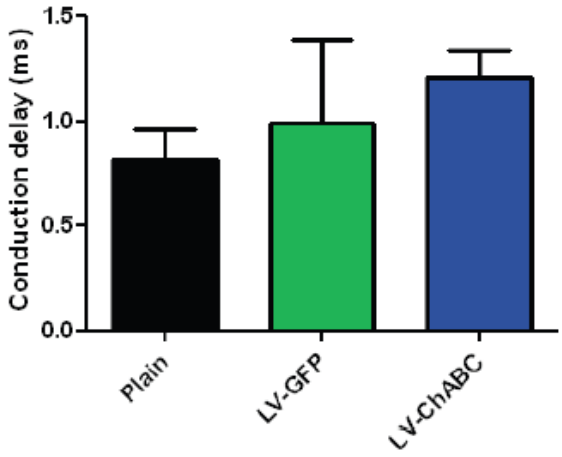

Conduction delay (ms)
1.5
1.0
0.5
0.0
Plain
LV-GFP
LV-ChABC

Figure 3.4: Changes in axonal conduction and conduction delay following contusion injury with or without LV-GFP or LV-ChABC treatment.

A: Conduction velocity was significantly slowed following contusion only (*=$p<0.01$ vs. below, n=5; two-way ANOVA) and did not improve following administration of LV-GFP or LV-ChABC ($p>0.05$, n=5 each group; two-way ANOVA). Data are shown as mean ± SEM. **B:** All experimental groups show a degree of conduction delay across the lesion, but this is not statistically different between groups ($p=0.522$, n=5 each group; one-way ANOVA).

treatment significantly improved conduction and LV-1 slightly improved conduction. This suggests that sustained and widespread CSPG digestion is required for a functional improvement to be realised.

Conduction velocity was measured for the two control groups (contusion only and LV-GFP) and the most efficacious ChABC-expressing viral vector (LV-3, hereafter referred to as LV-ChABC). Following contusion only, fibres that retained the ability to conduct across the lesion had significantly slowed conduction velocity compared to those activated by stimulating below the lesion (Fig. 3.4A; 19.5m/s above compared with 27.3m/s below; $p<0.01$). This suggests that these fibres are not functioning normally, despite retaining the ability to conduct action potentials. Neither the LV-GFP group nor the LV-ChABC group showed any improvement in conduction velocity (Fig. 3.4A; conduction velocity above the lesion 21.3m/s and 18.2m/s for LV-GFP and LV-ChABC, respectively; $p>0.05$).

Conduction delay across the lesion was calculated by measuring the difference in expected latency based on conduction velocity below the lesion and actual latency. There was no significant difference in conduction delay across the lesion between rats that received contusion only and those that were treated with LV-GFP and LV-ChABC (Fig. 3.4B; conduction delay across the lesion 0.82±0.1ms, 0.99±0.4ms and 1.21±0.1ms for contusion only, LV-GFP and LV-ChABC, respectively; $p>0.05$).

3.3.4 *Effect of LV-ChABC on spinal reflexes below the injury*

The effect of LV-ChABC treatment on the magnitude of response of two hindlimb nerves upon sural nerve stimulation at supramaximal C-fibre intensities was investigated. In these experiments LV-ChABC was compared with the control vector,

LV-GFP. Constant, repeated stimulation of the sural nerve elicited the wind-up phenomenon, in which the number of spikes evoked increases with successive stimuli. As described for brachial nerves in the previous Chapter, 25 high intensity impulses delivered to the sural nerve led to increasing bursts of activity in two hindlimb flexor nerves, with activity reaching a plateau after approximately 10 impulses. This pattern of increasing discharge of flexor motoneurons is known as wind-up. Both nerves studied, the motor nerve to biceps femoris and the common peroneal, displayed stereotypical wind-up responses but the magnitude of the reflex differed between the two nerves. The number of spikes recorded at baseline was typically 5-10 for the biceps femoris but much higher in the common peroneal, with approximately 100 spikes per second recorded. The maximum number of spikes elicited also varied: approximately 300 for the biceps femoris and 1300 for the common peroneal. The pattern of nerve activity was nevertheless very similar for both nerves (Fig. 3.5A, B). Upon area under the curve quantification, there was no difference in the magnitude of wind-up elicited in the motor nerve to biceps femoris between the two treatment groups (Fig. 3.5C; effect of treatment: $p>0.05$) or in the common peroneal nerve (Fig. 3.5D; effect of treatment: $p>0.05$).

The response of the nerves to sural nerve stimulation at A-fibre thresholds was also investigated. These low intensity stimuli (50-300μA, 100μs), delivered at the same frequency as a wind-up trial, do not provoke wind-up in naïve animals. The advantage of studying these lower threshold responses is that they allow us to see effects that may be obscured or saturated during higher intensity stimulation. In these experiments we did indeed see a clear difference between the treatment groups, as demonstrated in spike frequency plots for both the biceps femoris (Fig. 3.6A, C, E) and common peroneal (Fig. 3.6B, D, F) nerves. There was a significant effect of treatment at every

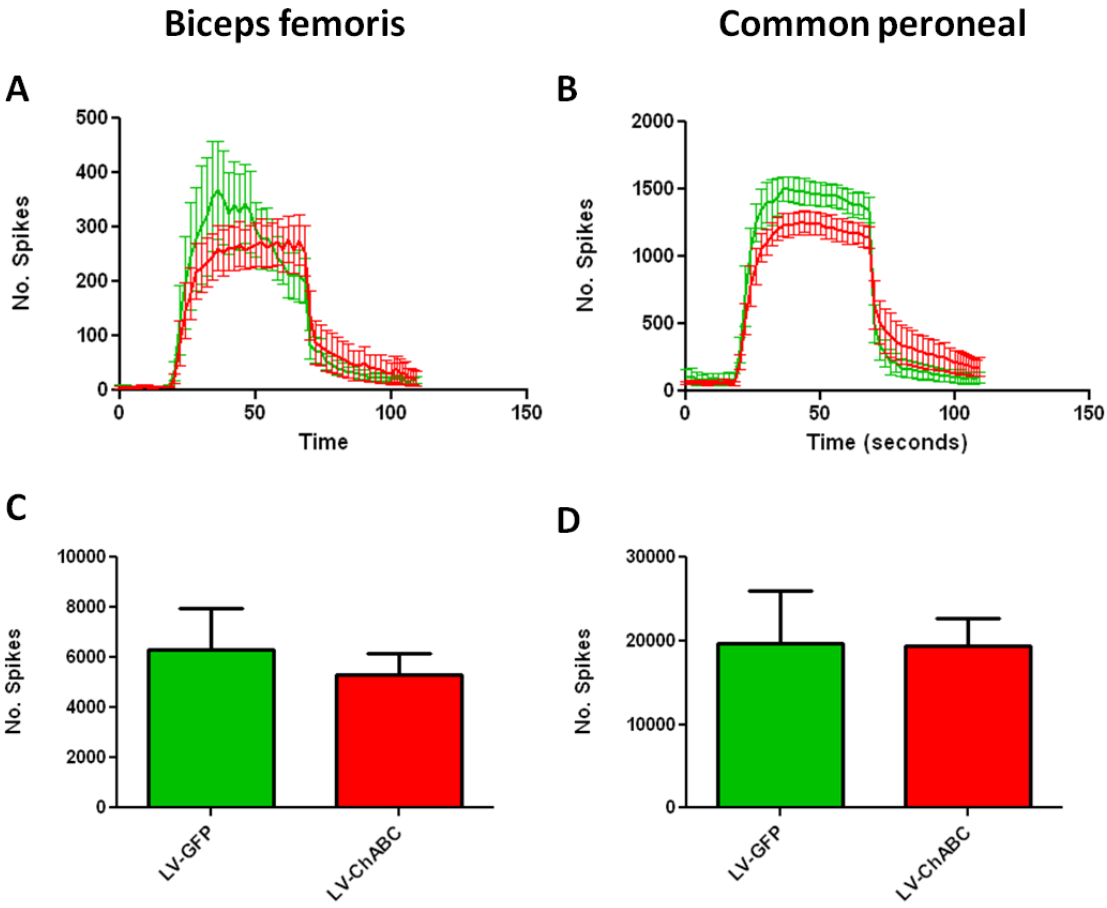

Biceps femoris
Common peroneal
A
B
C
D
No. Spikes
Time
Time (seconds)
LV-GFP
LV-ChABC

Figure 3.5: C-fibre wind-up after contusion injury and LV-ChABC or LV-GFP treatment. Spike frequency plots showing the number of spikes recorded from the biceps femoris (left panel) or common peroneal (right panel) per second before (0-20 seconds), during (20-70 seconds) and after (70-120 seconds) a wind-up trial. **A**, **B**: 25 stimuli of supramaximal C-fibre threshold were applied to the sural nerve (amplitude: 4mA, pulse width: 1ms). **A**: Baseline levels of activity in the biceps femoris are low but during the stimulating period a dramatic increase in activity is observed, reaching a peak after around 10 stimuli. The pattern and magnitude of wind-up is very similar between the treatment groups. **B**: Baseline activity in the common peroneal is higher than in the biceps femoris, but still stable at rest. During a C-fibre strength wind-up trial activity increases dramatically. The pattern of a rapid increase in activity, followed by a gradually decaying plateau is remarkably similar in the LV-GFP and LV-ChABC groups, with the LV-GFP group reaching a slightly higher plateau. **C**, **D**: The magnitude of wind-up elicited by C-fibre strength stimulation is not significantly different between the LV-GFP and LV-ChABC treatment groups in the biceps femoris (**C**) or common peroneal (**D**) nerves upon area under the curve quantification, which takes baseline activity and input value into account ($p>0.05$, $n=5$; unpaired t-test). Data are shown as mean ± SEM.

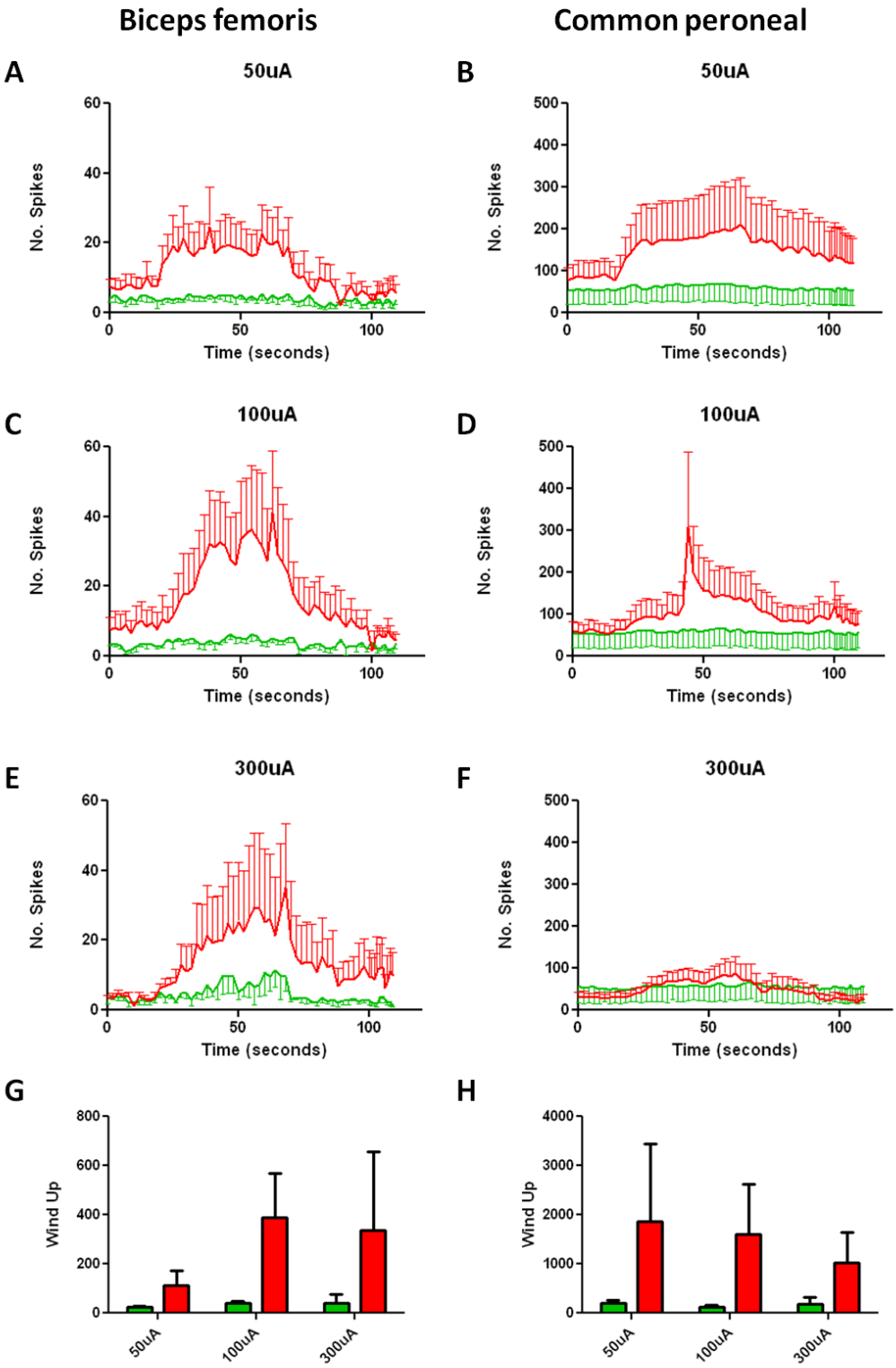

Biceps femoris
Common peroneal
A
50uA
B
50uA
C
100uA
D
100uA
E
300uA
F
300uA
G
H
No. Spikes
Time (seconds)
Wind Up
50uA
100uA
300uA

Figure 3.6: A-fibre wind-up after contusion injury and LV-ChABC or LV-GFP treatment. A-F: trials consisting of 25 stimuli of A-fibre strength were also applied to both nerves. The pulse width used was 100μs and the amplitudes were 50μA (**A**, **B**), 100μA (**C**, **D**) and 300μA (**E**, **F**). LV-GFP treated animals did not display wind-up responses in either nerve to any amplitude of A-fibre strength stimulus. In contrast, wind-up pattern responses were observed in both nerves of LV-ChABC animals at all amplitudes. The differences between LV-GFP and LV-ChABC are significant at all stimulus amplitudes, apart from 300μA in the common peroneal (effect of treatment: 50μA: $p<0.0001$ for both nerves; 100μA: $p<0.0001$ for both nerves; 300μA: $p<0.001$ for biceps femoris, $p=0.11$ for common peroneal, n=5, two-way RM ANOVA with Bonferroni post-hoc). **G**, **H**: Area under the curve quantification of spike discharge during a train of stimuli at A-fibre strength shows that LV-GFP treated animals display very small wind-up responses, but a trend towards larger responses are observed after LV-ChABC treatment, in both the biceps femoris (**G**) and common peroneal (**H**) nerves ($p=0.09$ for biceps femoris; $p=0.08$ for common peroneal; n=5, two-way ANOVA).

stimulus amplitude studied, apart from the common peroneal at 300uA (50uA: $p<0.0001$ for both nerves; 100uA: $p<0.0001$ for both nerves; 300uA: $p<0.001$ for biceps femoris, $p=0.11$ for common peroneal). LV-GFP treated animals never demonstrated wind-up like responses after A-fibre intensity stimulation, but some LV-ChABC treated animals (3 out of 5) displayed wind-up like responses in both nerves. Area under the curve quantification of wind-up at A-fibre strength did not reach significance (Fig. 3.6G, H; effect of treatment: $p=0.09$ for biceps femoris, $p=0.08$ for common peroneal). This is most likely due to two of the LV-ChABC treated animals that did not display wind-up upon low intensity stimulation.

3.3.5 *Pain behaviour after contusion injury and LV-ChABC treatment*

Wind-up is a normal response to noxious inputs. However, its appearance in contused animals upon A-fibre stimulation could potentially indicate an abnormal pain response. Therefore, the hindpaws of injured rats were tested for hypersensitivity to non-noxious mechanical and noxious thermal stimuli. No significant differences in withdrawal threshold to mechanical stimuli (Fig. 3.7A; $p>0.05$) or in latency to withdraw from a thermal stimulus were detected between the three treatment groups (Fig. 3.7B; $p>0.05$). These findings indicate that all animals have the motor ability to withdraw the hindpaw from a sensory stimulus, and also that long lasting, widespread CSPG digestion delivered after contusion injury does not cause pain in the hind paws.

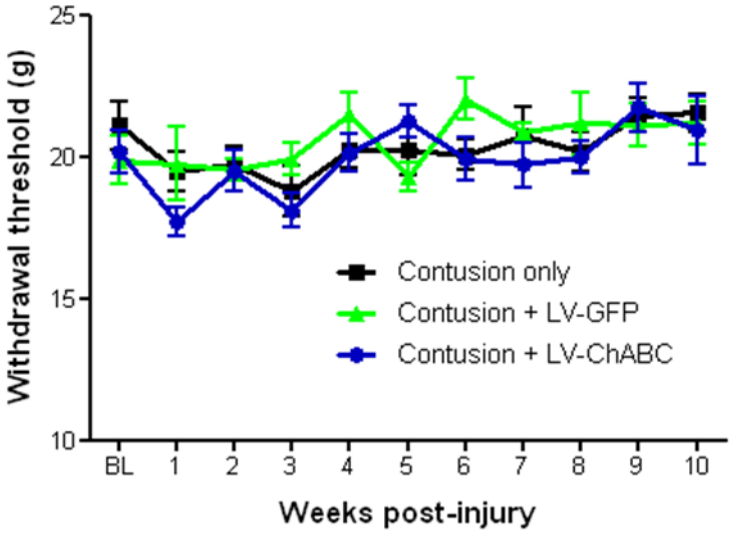
3.3.6 *Effect of LV-ChABC on behavioural function*

Animals were tested for performance in hindlimb locomotor function on two different tests. BBB scoring assesses gross locomotor function and the horizontal ladder is a skilled task that requires integration of sensory and motor skills. Before injury, all

animals achieved a baseline BBB score of 21, which indicates normal hindlimb locomotion and consistent forelimb-hindlimb coordination. Two days after injury animals in the contusion only group were significantly impaired, with mean a BBB score of 5.38±0.9. This score represents extensive movements of at least two hindlimb joints, but the full range of movement is not achieved and there is no weight support or stepping. Both LV treated groups were similarly impaired at this early time-point (Fig. 3.8A; 5.27±0.8 and 6.68±0.8 for LV-GFP and LV-ChABC, respectively; $p>0.05$). Over the 10 week testing period animals in all three groups achieved improved BBB scores, with the degree of improvement reaching a plateau by 3 weeks after injury. There was no significant difference in the scores achieved between the treatment groups over time (Fig. 3.8A; $p>0.05$), with animals reaching a plateau around a score of 13. This result signifies that, following a moderate contusion injury, animals in all treatment groups are similarly impaired after injury and partially recover at the same rate and to the same extent on this test of motor function.

At baseline testing rats performed very well on the horizontal ladder test, making errors only very rarely. After contusion injury animals were not tested on this task until they could take weight-supported steps, which meant the first post-injury assessment was at 1 week post-injury. At this time point animals in both control groups were severely impaired, making many footslips, and although there was some spontaneous recovery, this reached a plateau around 4 weeks post-injury and rats remained significantly impaired throughout the 10-week testing period (Fig. 3.8B). However, the group that received LV-ChABC treatment made significantly fewer errors during the task, as early as 1 week after injury (footslips at 1 week: 33.1±3.5 for LV-ChABC compared with 48.8±3.5 and 47.1±3.9 for LV-GFP and contusion only, respectively; $p<0.01$). This group then continued to improve and performed significantly better than controls into

A


Withdrawal threshold (g)
25
20
15
10
BL
1
2
3
4
5
6
7
8
9
10
Weeks post-injury
Contusion only
Contusion + LV-GFP
Contusion + LV-ChABC

B

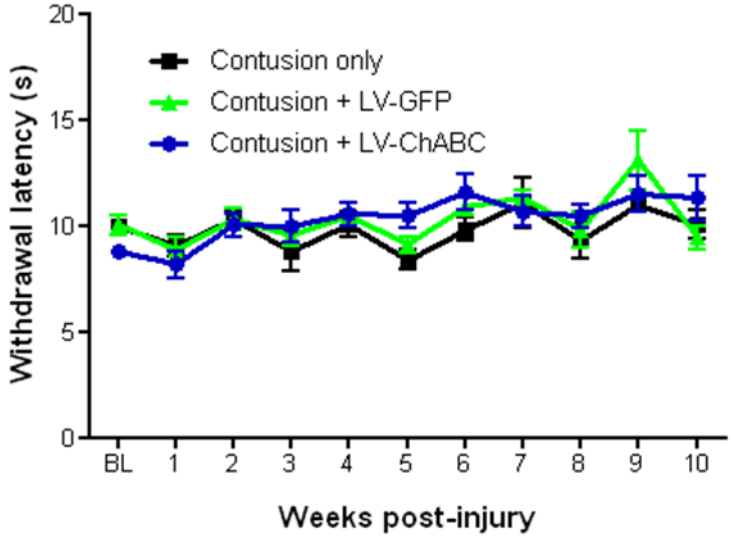

Withdrawal latency (s)
20
15
10
5
0
BL
1
2
3
4
5
6
7
8
9
10
Weeks post-injury
Contusion only
Contusion + LV-GFP
Contusion + LV-ChABC

Figure 3.7: LV-ChABC does not cause pain in the presence of a contusion injury. Animals did not develop hindpaw hypersensitivity as assessed by sensory testing of mechanical (**A**) or thermal (**B**) thresholds to withdraw over the course of 10 weeks following injury and treatment. There was no significant difference between groups ($p>0.05$, n=7 contusion and LV-ChABC, n=8 LV-GFP; two-way RM ANOVA).

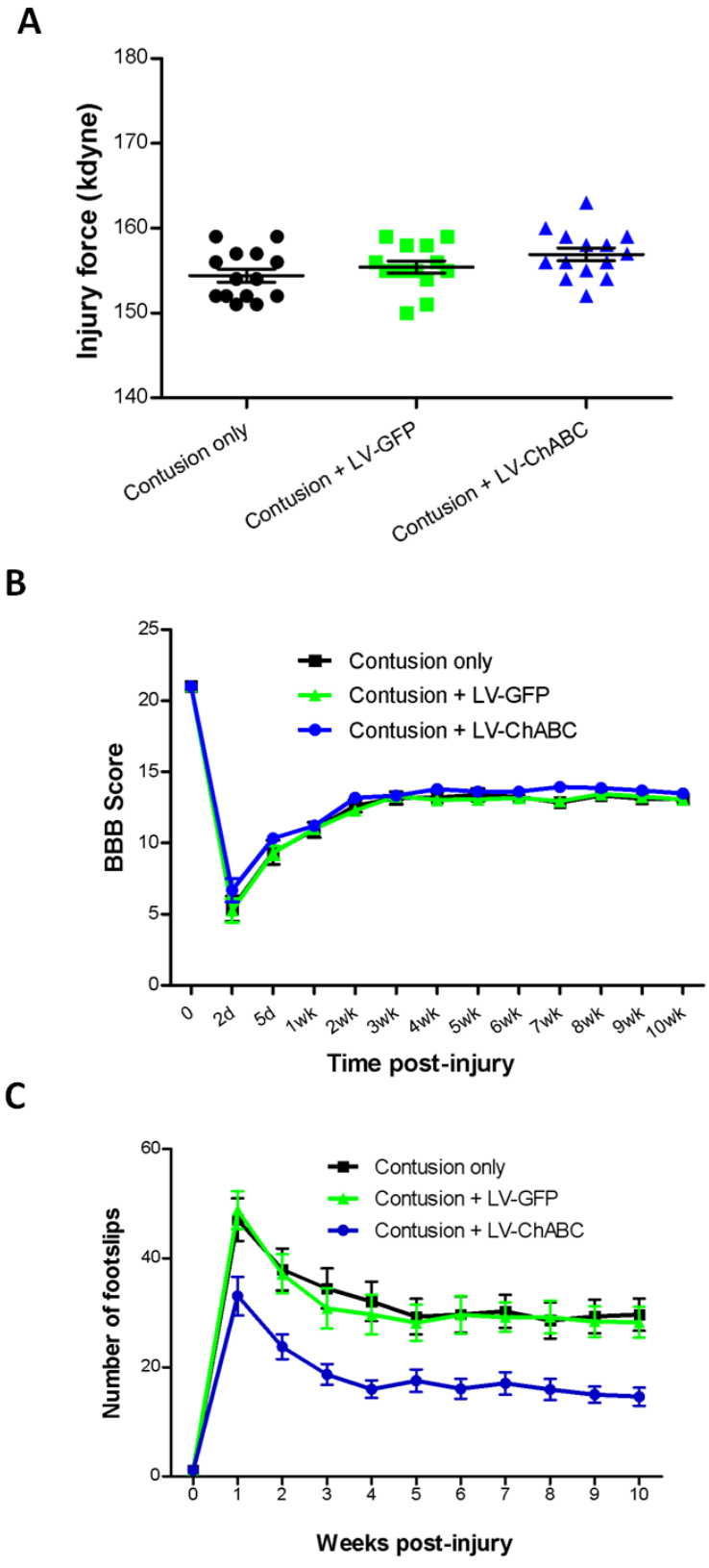

A
Injury force (kdyne)
180
170
160
150
140
Contusion only
Contusion + LV-GFP
Contusion + LV-ChABC
B
BBB Score
25
20
15
10
5
0
Contusion only
Contusion + LV-GFP
Contusion + LV-ChABC
0
2d
5d
1wk
2wk
3wk
4wk
5wk
6wk
7wk
8wk
9wk
10wk
Time post-injury
C
Number of footslips
60
40
20
0
Contusion only
Contusion + LV-GFP
Contusion + LV-ChABC
0
1
2
3
4
5
6
7
8
9
10
Weeks post-injury

Figure 3.8: Effect of LV-ChABC on behavioural function. A: BBB locomotor rating scores show a severe loss of function in all groups during the acute post-injury time period. Subsequently, both LV-ChABC treated and control groups display improvement, reaching a plateau ~3 weeks after injury, with a BBB score of ~13 (signifying frequent/consistent stepping but inconsistent forelimb/hindlimb coordination). **B**: As with the BBB test, animals that received no treatment or LV-GFP treatment are severely impaired on the horizontal ladder in the acute injury period but improve over time, reaching a plateau ~5 weeks after injury. LV-ChABC treated animals are not as severely impaired acutely after contusion injury, making ~15 fewer footslips at one week than untreated or LV-GFP treated animals. They similarly improve over time, reaching a plateau at ~4 weeks post-injury (*=$p<0.01$ vs. LV-GFP; n=15 contusion only and LV-ChABC, n=16 LV-GFP, two-way RM ANOVA with Bonferroni post-hoc). **C**: Force readouts from the contusion device show that the actual force delivered by the impact probe was not significantly different between groups ($p>0.05$, n=14 for contusion only, n=14 for LV-GFP, n=14 for LV-ChABC; one-way ANOVA).

the chronic post-injury period (Fig. 3.8B; footslips at 10 weeks: 14.6±1.6, 28.3±2.8 and 29.7±3.0 for LV-ChABC, LV-GFP and contusion only, respectively; $p<0.01$). Since all animals were equally impaired at 2 days post-injury in terms of BBB score, it is unlikely that LV-ChABC treated animals received milder injuries than controls. In addition, force readouts from the contusion device, which reflect the actual force delivered by the impact probe, were recorded at the time of injury for all animals. Force readout values for individual animals indicated that injuries were consistent and reproducible between groups (Fig. 3.8C; $p>0.05$). These results indicate that ChABC treatment can lead to a long-lasting functional improvement in the horizontal ladder, a task requiring sensorimotor integration.

3.3.7 *LV-ChABC treatment affects lesion pathology*

Contusion injury leads to destruction of spinal grey matter resulting in a cystic cavity in the lesion epicentre, surrounded by a rim of spared white matter. A typical section from the lesion epicentre of an animal that received contusion only is shown in Fig. 3.9A, stained for myelin with the lipophilic eriochrome cyanine stain. The spinal cord has a large central cavity with a rim of spared white matter. Following treatment with LV-ChABC, cavitation at the lesion epicentre at a chronic stage (10 weeks) was dramatically decreased compared to LV-GFP and contusion only animals (Fig. 3.9A-C). Quantification of the size of the cavity and its rostrocaudal extent was obtained by measuring cavity size at 600µm intervals rostral and caudal to the lesion epicentre up to a distance of 3.6mm in either direction, confirming the striking reduction in cavity size (Fig. 3.9D). In order to investigate the nature of glial scar, the injured spinal cords were immunostained for GFAP. After contusion only, a thick border of reactive astrocytes forms around the lesion, and here we observe intense GFAP expression (Fig. 3.9E). In

contrast, the gliotic border of the injury was very different in LV-ChABC treated animals. GFAP expression is more diffuse, with elongated astrocytic processes extending into the lesion cavity (Fig. 3.9F). Although there is a rim of spared CNS tissue after a contusion injury, the remaining tissue is typically white matter, with destruction of grey matter and death of most neurons at the lesion epicentre. To investigate whether LV-ChABC could lead to better survival of neurons within the lesion, immunostaining for NeuN was performed. Indeed, a greater number of neurons appeared to survive the injury, as demonstrated by expression of a neuronal cell body marker, NeuN (Fig. 3.9E, F). These results demonstrate amelioration of much of the pathology classically observed following moderate contusion injury by LV delivery of ChABC.

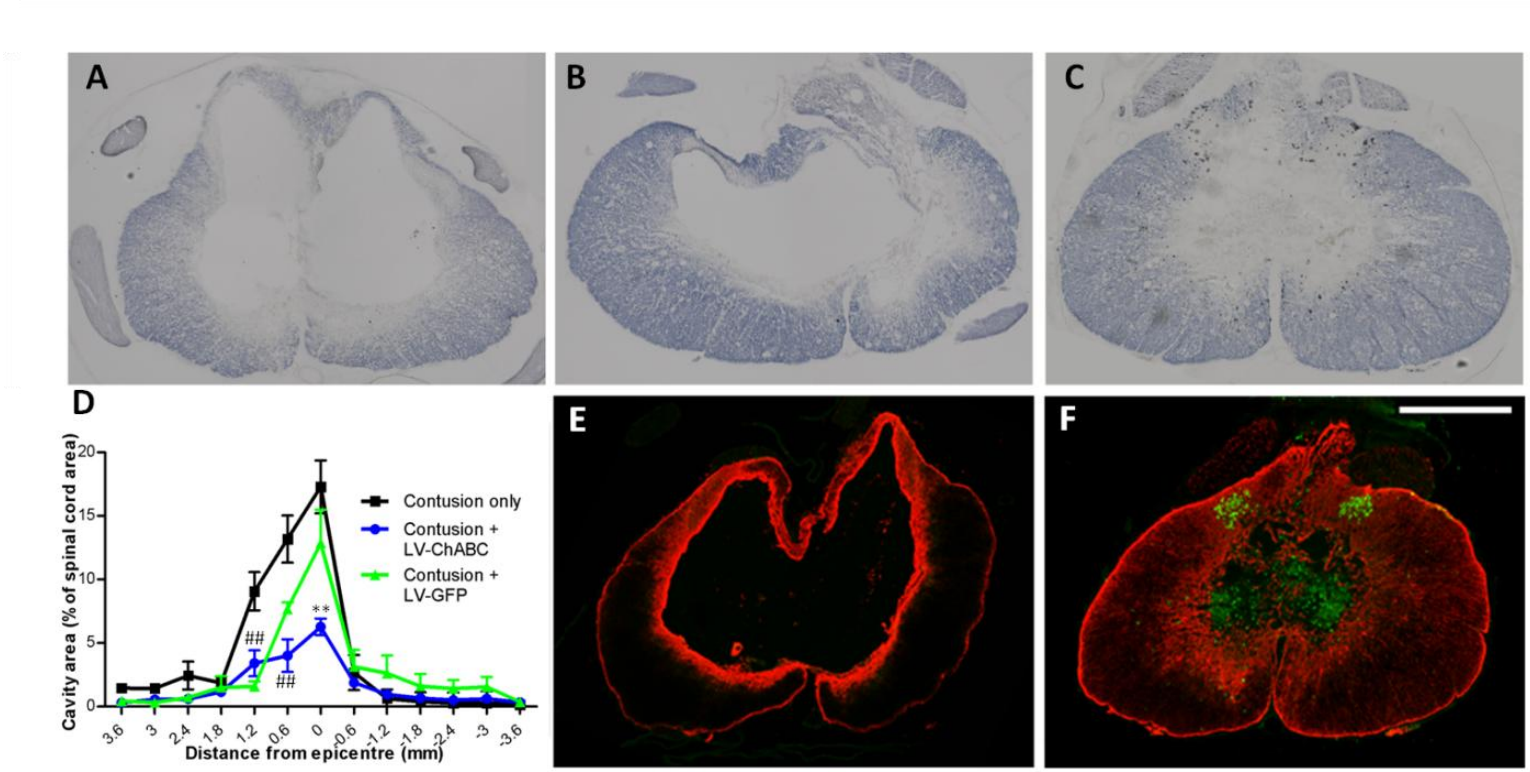

A
B
C
D
E
F
Cavity area (% of spinal cord area)
20
15
10
5
0
3.6
3
2.4
1.8
1.2
0.6
0
-0.6
-1.2
-1.8
-2.4
-3
-3.6
Distance from epicentre (mm)
Contusion only
Contusion + LV-ChABC
Contusion + LV-GFP
##
##
**

Figure 3.9: LV-ChABC treatment ameliorates lesion pathology after contusion injury. A-C: transverse spinal cord sections from the lesion epicentre were stained with the eriochrome cyanine stain for myelin. At 10 weeks post-injury the epicentre of spinal cords that received contusion only (**A**) or LV-GFP (**B**) had large central cavities surrounded by a spared rim of white matter, while following LV-ChABC treatment, cavitation was much reduced (**C**). **D**: Cavity size was measured at 600µm intervals for 3.6mm rostral and caudal to the lesion epicentre. LV-ChABC treatment led to a significant decrease in cavity size compared to contusion only and LV-GFP (**=$p<0.001$ vs. contusion only and LV-GFP, ##=$p<0.001$ vs. contusion only; n=4; two-way RM ANOVA with Bonferroni post-hoc). **E**, **F**: transverse spinal cord sections from the lesion epicentre were stained for GFAP (red) and NeuN (green). After contusion only a border of intense GFAP expression surrounds the lesion cavity and no NeuN positive cells are visible at the lesion epicentre (**E**). After LV-ChABC treatment GFAP expression is more diffuse, the lesion border less sharply defined and NeuN positive cells are visible near the dorsal surface of the spinal cord (**F**). Scale bar = 1mm.

3.4 Discussion

This study has shown the efficacy of LV-ChABC in a clinically relevant experimental model of traumatic spinal cord injury. By using a gene therapy approach, we were able to deliver ChABC chronically to the spinal cord. This resulted in a long-lasting degradation of CSPGs and in remarkable improvements in lesion pathology, including a significantly reduced degree of cavitation and an altered glial scar. Functional recovery was demonstrated by improved performance on a behavioural task and improved spinal conduction. In addition, reflexes studied below the level of the injury demonstrated the plastic potential of the LV-ChABC treated CNS.

3.4.1 *Gene delivery of ChABC*

We have used an LV to deliver ChABC to the rat spinal cord. We were able to achieve widespread and long-lasting expression of ChABC, with digested CSPGs observed several spinal segments away from the site of injection and persisting over many weeks. Other studies of ChABC after contusion injury have used multiple administrations or continuous infusion of enzyme to see an effect (Caggiano et al., 2005; Karimi-Abdolrezaee et al., 2010). Improvements in performance on the horizontal ladder task were apparent as soon as 1-2 weeks after injury, consistent with the extensive digestion of CSPG by 2 weeks post LV-ChABC injection, as visualised by C-4-S expression. The importance of the degree of CSPG digestion on functional outcome is illustrated by measurements of spinal conduction. Treatment with either LV-2 or bacterial ChABC enzyme, both of which produce a localised area of CSPG digestion (Fig. 3.1B), did not lead to any improvement in axonal conduction. LV-3 treated animals, in contrast, which displayed a very widespread and sustained area of digestion, showed a large and significant increase in the number of dorsal column fibres conducting through the

conduction site. LV-1 treatment, which led to an intermediate amount of digestion, displayed an increased but non-significant improvement in conduction. Therefore, the amount of CSPG digestion appears to correspond with the degree of improvement in conduction. We have used an efficient method to deliver ChABC to the spinal cord over a chronic time period, thereby overcoming the problems of delivering an enzyme, which is liable to be degraded after a relatively short period of activity. The use of viral vectors as gene therapy agents is promising, especially considering improvements in the safety profile of LVs, such as the use of self-inactivating vectors. However, significant challenges remain to be addressed. Integration into the host genome can potentially result in oncogene activation or inactivation of tumour suppressor genes, and responses of the immune system may be detrimental (Matrai et al., 2010). This Chapter reports high level, sustained transgene expression, but long-term CSPG digestion may lead to unforeseen and undesirable consequences. A possible solution may be the regulation of viral transgene expression and the use of inducible or tissue-specific promoters and excisable transgenes are currently under development and will improve the translational potential of viral vectors (Goverdhana et al., 2005; Bouard et al., 2009). First, more detailed knowledge of the appropriate level and time period of ChABC expression is required. Nevertheless, LV-mediated ChABC delivery remains an attractive candidate for future application in SCI.

3.4.2 *Effects of LV-ChABC on lesion pathology*

A moderate severity contusion injury leads to a local loss of neuronal cell bodies, destruction of grey matter, progressive cavity formation and reactive gliosis. In these studies we observed a reduction in all of these pathological features after LV-ChABC treatment. At acute and sub-acute time points (1 day to 2 weeks following injury) cavity

area at the lesion epicentre is reported to be approximately 10% of total cord area, which progressively increases over time to 30% by 12 weeks after injury (James et al., 2011). Similarly, in the present study cavity size in contused animals with no treatment was large by 10 weeks post-injury. In contrast, contused animals treated with LV-ChABC had a significantly smaller cavity area at the lesion epicentre and the appearance of the cord resembled a lesion arrested at an earlier stage, such as 1-2 weeks post-injury. Clearly, a smaller cavity represents a greater amount of potentially viable neural tissue. Indeed, we showed a significant increase in conduction in the dorsal column pathway following LV-ChABC treatment (discussed below), possibly indicating a neuroprotective effect of the treatment. Animals that had received LV-GFP injections also had a significantly larger cavity at the epicentre of the injury, but the rostrocaudal spread of the cavity is less than that of contusion only animals. This finding illustrates the importance of including suitable control treated animals. Administering an intraspinal injection of both ChABC and GFP-expressing vectors involves an extra ten minutes of anaesthesia, opening of the dura, insertion of a needle into the parenchyma of the cord and exposure of nervous system tissue to a viral vector. Any of these factors could contribute to the observed reduction in cavity size, compared to uninjected animals. For example, opening the dura could relieve a build up of pressure due to oedema and haemorrhage after contusion, leading to ameliorated ischaemia and improved lesion pathology. It is important to note that the LV-GFP animals did not display improved behavioural and electrophysiological outcomes.

Another pathological difference between LV-ChABC and control groups was the appearance of the glial scar. Following contusion injury CSPGs are dramatically upregulated at the level of the lesion and reactive gliosis progressively becomes more focussed around the cavity perimeter to form a dense glial scar (James et al., 2011). In a

similar manner to cavity size discussed above, gliosis at a chronic time point in LV-ChABC treated animals resembled an earlier, sub-acute time point. Reactive astrocytes were present in the cord, but were expressed more diffusely around the cavity and had a more elongated morphology. This phenomenon is reminiscent of the astrocytic response in the hypothalamic arcuate nucleus, where the resident neuropeptide Y containing neurons regenerate much more readily than elsewhere in the CNS. Following transection of NPY neurons the glial scar is less fibrotic in nature, shows astrocytic perikarya which are less closely apposed and connected by fewer gap junctions; axons are also able to project into the scar more readily (Alonso and Privat, 1993). This study suggests that altering the nature of the glial scar may affect outcome, in agreement with the present study.

3.4.3 *Changes in axonal conduction following LV-ChABC treatment*

The percentage of sampled fibres found to be conducting through a contusion injury 10 weeks after injury and no treatment was found to be approximately 10% of pre-injury levels. This finding is in agreement with others who have reported a 90% decrease in dorsal column compound action potential amplitudes across a thoracic contusion injury (Hains et al., 2004) and consistent with conduction failure after moderate contusion injury (James et al., 2011). A number of contributing factors are likely to be responsible for this decreased conduction. Oligodendrocytes undergo apoptosis as a result of SCI and contribute to chronic demyelination, which may result in spinal cord dysfunction (Blight, 1993; Liu et al., 1997; Beattie et al., 2000), including conduction block (McDonald and Sears, 1969; Shuman et al., 1997). The inflammatory response starts early after injury and involves the release of numerous factors, including nitric oxide (NO), which can cause conduction block (Redford et al., 1997). It is very likely that

CSPGs play a part in conduction failure as they are dramatically upregulated after injury and this increased expression persists for long periods after the initial trauma (Jones et al., 2003a; Tang et al., 2003; Iaci et al., 2007). Indeed, it has recently been shown that CSPGs block conduction, either when upregulated after injury or when applied directly to the spinal cord (Hunanyan et al., 2010). In this Chapter we describe a 2.5 fold increase in conduction across a contusion injury when CSPG expression is chronically suppressed by administration of LV-ChABC, supporting the hypothesis of a role for CSPGs in conduction failure. The mechanism underlying this conduction block is currently unknown but a prominent CSPG, NG2, was found by Hunanyan et al. (2010) to block conduction more potently than other CSPGs. Since NG2 accumulates at Nodes of Ranvier (Butt et al., 1999; Martin et al., 2001), it is possible that it mediates nodal dysfunction after SCI.

The conduction velocity of fibres projecting through the lesion was found to be significantly slower than those below, indicating that these fibres are not functioning normally and this is likely to be due to demyelination of surviving axons (Arvanian et al., 2009). LV-ChABC treated animals did not exhibit any increase in conduction velocity, so the improved conduction is probably not due to enhanced remyelination. Rather, it could be due to lifting of the conduction block by another mechanism, such as reversal of nodal dysfunction. ChABC has been reported to promote regeneration of axons after injury (Bradbury et al., 2002; Houle et al., 2006) and a population of newly regenerated, thinly myelinated axons could have led to our observed results with LV-ChABC treatment (i.e. an increase in the number of fibres conducting above the lesion, but with a slower than normal conduction velocity). Since LV-ChABC treated animals exhibit improved sensorimotor function on the ladder test at very early stages after injury (1-2 weeks), it is more likely that the increase in conduction is due to sparing of

axons rather than regeneration. This hypothesis is supported by the white matter sparing seen upon eriochrome cyanine staining. A candidate population of viable, non-conducting spared fibres were recently identified (James et al., 2011) and it may be this population of axons whose conduction is improved by ChABC. To definitively answer the question of regeneration versus spared neurons it will be necessary to look at conduction above and below the injury at early stages after injury, when behavioural effects are already apparent but regeneration could not already have occurred. In addition, axonal tracing of dorsal column axons could be performed and would provide evidence of any regenerating axons. It is important to note that in this study axonal conduction was measured in the dorsal column system, but the anatomical changes suggest that multiple ascending and descending pathways may be similarly protected by ChABC.

It is known from studies in the field of demyelinating disorders that a major cause of permanent neurological deficit in models of multiple sclerosis is axonal degeneration (Trapp et al., 1998; Ganter et al., 1999) and the major risk factors for axonal death are the presence of inflammation and remyelination (Raine and Cross, 1989; Ferguson et al., 1997; Trapp et al., 1998), both of which are prominent features in the environment of a spinal cord injury (Donnelly and Popovich, 2008; James et al., 2011). This increased vulnerability to degeneration can be ameliorated by strategies that protect neurons (Bechtold et al., 2005; Bechtold et al., 2006; Black et al., 2006). Several treatments have aimed to target the secondary phase of damage after SCI, particularly by attempting to minimise damage due to the inflammatory response (Young, 1991; Oudega et al., 1999; Popovich et al., 1999). More recently, it has been shown that ChABC also has neuroprotective effects in long-range projection neurons (Carter et al., 2008; Carter et al., 2011) and this is supported by our observation of an increase in

NeuN-positive cells at the lesion epicentre, suggesting sparing of neurons in LV-ChABC treated animals.

Thus, the observed improvement in axonal conduction in the present study may be due to mitigation of the CSPG-induced conduction block and/or a neuroprotective effect of ChABC, leading to sparing of axons. Our data suggest that regeneration of injured axons may not be the leading cause of improved conduction, but this possibility cannot be ruled out until further study.

3.4.4 *Plasticity of reflexes below the level of the lesion*

Repeated stimulation of a peripheral nerve at C-fibre strength leads to a progressive increase in activity of dorsal horn neurons (Mendell and Wall, 1965) and in flexor motoneurons (Schouenborg and Sjolund, 1983), as described in the previous chapter. Here we have examined this phenomenon, known as wind-up, by stimulating and recording from branches of the sciatic nerve in the hindlimbs of rats with thoracic contusion. As we were stimulating and recording from branches of the sciatic nerve, spinal circuitry involved in these reflexes (largely L4-L5 spinal segments) should be structurally intact. Indeed, organised locomotor EMG patterns can be recorded from both cats (Duysens and Pearson, 1980; Andersson and Grillner, 1983; Pearson, 1995) and humans (Dietz et al., 1995; Harkema et al., 1997; Dietz et al., 2002) following SCI. However, inflammatory processes after thoracic SCI can cause changes at the lumbar level (Detloff et al., 2008; Garcia-Alias et al., 2010) and the loss of supraspinal input can cause spinal neuronal dysfunction (Dietz, 2010). The effect of a moderate severity contusion injury on wind-up of flexor motoneurons in peripheral nerves upon supramaximal stimulation was investigated. There was no difference in C-fibre wind-up

magnitude between LV-GFP and LV-ChABC treated groups. This is consistent with findings in the previous Chapter that, when applied to intact spinal circuits associated with intact peripheral nerves, ChABC does not elicit an exaggerated wind-up response. These findings are in disagreement with a recent publication reporting that sciatic nerve wind-up responses increase after spinal injury (Castro et al., 2011). An important difference between the two studies is that Castro et al. (2011) found that injured animals exhibited hypersensitivity of the hindpaws in association with elevated wind up responses, whereas we did not, suggesting that hyperexcitability of spinal reflexes and below-level pain are connected.

In addition to studying classic C-fibre mediated wind-up, we measured hindlimb nerve responses to repetitive stimuli at A-fibre intensity. In naive animals, wind-up responses can only be elicited by the stimulation of primary afferent C-fibres and not by Aβ fibre stimulation alone (Schouenborg and Sjolund, 1983; Schouenborg, 1984; Weng et al., 1998). As expected, A-fibre stimulation did not evoke wind-up of flexor motoneurons of LV-GFP treated rats. Conversely, some LV-ChABC treated animals did begin to display wind-up responses and these data, although area under the curve quantification did not reach the level of statistical significance, showed a clear trend towards an effect of treatment and future work will be necessary to validate these results. The wind-up responses were observed even at the lowest stimulation paradigm, meaning that it is unlikely that we are recruiting C-fibres with lowered activation threshold and rather that this is genuine A-fibre mediated wind-up. This phenomenon has been previously described in the context of hyperalgesic states induced by peripheral nerve injury or inflammation (Thompson et al., 1995) and as spontaneous below-level pain is a feature of clinical and experimental SCI (Finnerup and Baastrup, 2012), the possibility that A-fibre wind-up could underlie this pain is raised, especially as wind-up of motoneurons

reflects spinal processing of nociceptive information. However, no evidence for heightened pain sensation was found in injured animals treated with LV-ChABC, upon behavioural testing. Together with the observation that C-fibre mediated wind-up is not different between the two treatment groups, these findings indicate that the emergence of this response may not indicate a pain state, but rather suggest that spinal neurons are more amenable to plastic change. As shown is Chapter 2, ChABC can potentiate the reorganisation of intact spinal circuits in the presence of a distant injury and wind-up is a form of short-term plasticity, so it may be that ChABC is increasing the plastic potential of spinal neurons. Indeed, it is possible to capitalise on the ability of spared neuronal circuits to reorganise through use-dependent mechanisms and train the isolated spinal cord (Dietz, 2010; Musienko et al., 2012). A recent study combining ChABC and rehabilitative training showed a strong synergistic effect of the two treatments (Garcia-Alias et al., 2009). The authors suggest that the application of a plasticity-promoting treatment leads to the formation of random *de novo* connections and that motor training selects the functional connections, eliminating inappropriate connections (Garcia-Alias and Fawcett, 2012). The combination of the plasticity promoting effects of ChABC and the refining influence of rehabilitative training may prove to be a powerful combination for future therapies.

3.4.5 *Effect of LV-ChABC on hindlimb function*

To investigate whether spinal ChABC treatment could lead to an improvement in hindlimb function we assessed the rats' behaviour on the BBB (Basso et al., 1995) and horizontal ladder (Metz and Whishaw, 2002) tests. The ladder test revealed improvements as early as 1 week after injury, and these were maintained throughout the testing period. The injury severity did not differ between groups, as shown by force

readouts, and animals had similar levels of impairment, as assessed by BBB locomotor scores. Therefore, these findings point to an early neuroprotective mechanism behind the improved performance. It is known that axonal injury of projection neurons leads to a negative effect on distant cell bodies, which die or enter a chronic state of atrophy (Egan et al., 1977; Barron et al., 1988; McBride et al., 1989; Wannier-Morino et al., 2008). ChABC mediated repair is usually attributed to its axonal regeneration and plasticity-promoting effects, but there have been recent reports that it may also have neuroprotective effects. Carter et al. (2008) showed that degradation of spinal CSPGs lead to a reversal of the atrophy of distant axotomised corticospinal cell bodies, indicating a retrogradely mediated protective effect. This finding is in agreement with other studies showing that suppression of collagenous or glial scarring at the site of an SCI by other mechanisms also leads to an amelioration of rubrospinal (Davies et al., 2006) and corticospinal (Klapka et al., 2005) neuronal cell atrophy. Changes in signalling pathways (e.g. PKC, ERK) involved in mediating neuronal survival have also been observed, adding further evidence to suggest a neuroprotective mechanism of ChABC mediated repair (Carter et al., 2008). Due to this very early effect of ChABC, it would be interesting to apply the treatment with a delay in order to investigate whether ChABC would be able to rescue ladder performance from a deficit as great as untreated animals. This would help to separate the early, neuroprotective effects from later regeneration or plasticity-mediated mechanisms of repair.

No differences between groups in performance on the BBB test were detected, with all animals plateauing at a BBB score of ~13. This appeared to be due to the failure of the animals to become consistently coordinated after injury, which means making no mistakes in open field locomotion at all, and no animals reached this hurdle (James et al., 2011). The nature of the BBB scoring system is that it is non-linear and exhibits

some considerable hurdles in moving from one point to the next at higher points in the scale. The test, while having the advantage of being widely used, may not be sensitive in some circumstances and may not recognise small improvements in function. Others have reported improvements in BBB scores following spinal contusion injury and various experimental interventions. However, these studies mostly used injury paradigms that were more severe so the separation between groups occurred at lower scores, where coordination is not yet considered (Jakeman et al., 1998; Karimi-Abdolrezaee et al., 2006; Brown et al., 2011; Cerri et al., 2012). The ladder test incorporates both sensory and motor ability and the improved conduction in sensory fibres observed during our electrophysiological experiments point to improvement in the sensory modality being a factor in the recovery of ladder function. Rat behaviour on the ladder test has been shown to correlate over time with electrophysiological improvements in conduction, but their performance on the BBB score has a lower correlation with improved conduction (James et al., 2011). As the BBB is a gross locomotor test and the ladder has a sensory component, this suggests that the sensory recovery in this model is important. However, more accurate elucidation of the roles of the different neural pathways could be achieved in the future by using additional behavioural tests that separate motor, sensory and proprioceptive functions. The failure of the animals to become consistently coordinated on the BBB test, for example, may be due to a combination of muscle weakness, poor proprioception and limited sensory feedback. A pure sensory or proprioceptive test may provide a better correlate for the electrophysiology used in these studies.

3.4.6 *Conclusion*

This study has shown clear evidence for the beneficial effect of ChABC treatment in a clinically relevant experimental model of spinal cord injury. We propose that long-lasting and widespread CSPG digestion, achieved by the gene delivery approach employed, is responsible for the preservation of neural tissue and significant improvements in behavioural and electrophysiological tests.

CHAPTER 4

Developing viral vectors for the study of anatomical plasticity in the nervous system

4.1 Introduction

Anatomical changes after central or peripheral nervous system injury have long been studied in order to provide insight into the structural basis for observed functional changes. A variety of methods have been used over the last century to this end. Cajal used silver staining methods to observe the response of the nervous system to various experimental lesions. It was Cajal, for instance, who first showed that damaged dorsal roots could regenerate axons towards but not into the spinal cord. He observed that when they reached the PNS/CNS border, the axons formed large retraction bulbs and growth stalled. Later, silver staining methods were refined to preferentially stain degenerating axons and this allowed the reaction of damaged axons to be studied specifically (Glees, 1946; Nauta and Gygax, 1951, 1954; Blackstad, 1965; Fink and Heimer, 1967). For example, Liu and Chambers applied this approach to study 'spared roots'. That is, they cut a number of dorsal roots above and below a target root (the spared root). Some time later the spared root itself was cut and the degenerating fibres stained to ask if they had expanded their central terminals after the initial lesion of their neighbouring roots (Liu and Chambers, 1958). The authors concluded that they had indeed expanded their central arbours. This conclusion was challenged on technical grounds – that the silver staining may have been confounded by staining of the tracts of the original lesioned axons or dense body impregnation of glia in the deafferented region (Rodin et al., 1983). Such concerns led to repeated efforts to improve selective anatomical marking of particular populations of neurons, and to the birth of the field of neuroanatomical tract tracing in the latter half of the 20th century. The application of these principles led to an explosion in our knowledge of the structure and organisation of the CNS, as well as the rapid development of additional tracers and this has been

extensively reviewed elsewhere (Kobbert et al., 2000; Vercelli et al., 2000; Lanciego and Wouterlood, 2011).

4.1.1 *Tract tracing molecules*

Kristensson and Olssen were the first to describe the ability of intrinsic cellular machinery to transport the enzymatic protein, horseradish peroxidase (HRP), thereby allowing visualisation of the origin, course and termination of groups of neurons (Kristensson and Olsson, 1971a, b). The development of this technique was a breakthrough in neuroanatomy, as it allowed the tracing of neuronal connections *in vivo*. This is a retrograde technique as application of HRP is internalised and transported back to the cell body, allowing identification of the axonal projection and the location of the parent cell. Around the same time, radiolabelled amino acids began to be used for neuroanatomical labelling (Cowan et al., 1972). The labelled amino acids are taken up by the cell body, incorporated into proteins and undergo axoplasmic transport. As the machinery for the manufacture of proteins is located in the cell body, this technique is an example of anterograde tracing, which involves the centrifugal transport of macromolecules along axons and is used to identify the targets of populations of neurons. Autoradiography (to reveal the location of the radiolabel) was most famously used to demonstrate ocular dominance columns (Hubel et al., 1977).

Subsequently, the arrival of fluorescent tracers led to the ability to label multiple pathways in different colours. The relative ease, sensitivity and reliability compared to autoradiography or degeneration techniques led to their widespread use and the rapid development of a plethora of additional dyes. Early dyes applied to retrogradely label neurons include Fast Blue (Kuypers et al., 1980) and Lucifer Yellow (Stewart, 1981), but these have been supplemented by Fluorogold (Schmued and Fallon, 1986), Mini

Ruby (Novikova et al., 1997) and fluorescently tagged microspheres (Cornwall and Phillipson, 1988). Anterograde tracers include DiI (Thanos and Bonhoeffer, 1983, 1987), the plant lectin *Phaseolus vulgaris* leucoagglutinin (Gerfen and Sawchenko, 1984; Schofield, 1990; Wouterlood et al., 1990b) and dextran amines (Gimlich and Braun, 1985; Glover et al., 1986; Nance and Burns, 1990; Schmued et al., 1990). Transganglionic tracers such as CTB and wheat germ agglutinin (Schwab et al., 1978) can also be used to visualise primary sensory neurons and their central projections when injected into a peripheral nerve. This Chapter focuses on two neuronal populations: the corticospinal tract (CST) and primary sensory neurons. As we propose a new tool for studying these tracts, the tracers that have been most commonly applied to investigate their projections, biotinylated dextran amine (BDA) and CTB, respectively, will be described in more detail.

BDA is part of the dextran amine family of molecules, which are hydrophilic polysaccharides that are resistant to cleavage by endogenous cellular glycosidases (Vercelli et al., 2000). The biotinylated form was found to be a superior anterograde marker and is the most commonly used dextran amine (Veenman et al., 1992). BDA is transported in axons at a rate of 15-20mm a week, is stable in rat neurons for at least four weeks and tolerates a range of fixing and processing techniques (Reiner et al., 2000). Upon immunolabelling, the BDA appears homogeneously distributed within the neuron, resembling Golgi silver impregnation. The feature that makes this tracer especially useful for labelling the CST is that it appears equally homogeneous even very long distances from the cell body (Lanciego and Wouterlood, 2011), which can be a number of centimetres in the rat.

The beta unit of the cholera toxin is non-toxic and is taken up after binding to the GM1 ganglioside (Trojanowski, 1983). CTB was found to lead to more sensitive retrograde labelling of neurons than HRP (Trojanowski et al., 1981), which is due to its superior uptake via adsorptive endocytosis, rather than the fluid-phase endocytosis relied upon by HRP and the slower rate of elimination (Lanciego and Wouterlood, 2011). Although it was first introduced as a retrograde tracer, it became apparent that CTB could also be transported efficiently in an anterograde direction (Angelucci et al., 1996). Combined with the high level of anatomical detail produced, this led to the widespread use of CTB as a transganglionic tracer. Despite these advantages, any tracer that relies on receptor-mediated uptake must be used with caution as receptors may be unevenly expressed across neuronal subtypes or act differently in the presence of damaged axons. This problem is illustrated by studies of large myelinated Aβ fibres, which are preferentially labelled by CTB (Robertson and Grant, 1985; LaMotte et al., 1991). After peripheral nerve injury, it was claimed that these fibres had sprouted into the superficial dorsal horn, which is normally exclusively innervated by nociceptive fibres, and that this sprouting was responsible for pain hypersensitivity (Woolf et al., 1992; Shortland et al., 1997). It was subsequently shown that CTB also labels unmyelinated fibres when applied to a transected nerve (Tong et al., 1999; Bao et al., 2002) and single cell tracing studies have found little evidence that Aβ fibres sprout into superficial laminae after injury (Hughes et al., 2003).

Thus, despite the clear benefits of injectable tracing molecules, important limitations do exist. Injury-induced changes in the axonal transport system can lead to apparent changes in tracer density, which can be misleading, and most commonly employed tracing molecules require immunohistochemistry or enzyme histochemistry, which precludes their use for *in vivo* imaging (Kobbert et al., 2000; Lanciego and Wouterlood,

2011). Another important limitation of injectable tracing molecules is the considerable variability in the number of neurons and/or axons labelled between animals.

4.1.2 *Intracellular dye injection*

Intracellular injection of dyes such as Lucifer Yellow (Buhl et al., 1989; Wouterlood et al., 1990a; Ochiai et al., 1993), HRP (Koerber et al., 1990; Pierce and Mendell, 1993), Neurobiotin (Kita and Armstrong, 1991) and biocytin (Coleman and Friedlander, 1992) are used to improve the quality of filling of neuronal processes and also to allow coupling of a neuron's morphological and electrophysiological characteristics. By injecting dye into a neuron after an intracellular neurophysiology session, the morphological features of the cell can be documented in addition to the electrophysiological characteristics. Despite the impressive level of anatomical detail revealed using intracellular dye injection, the disadvantages of this procedure are the technical difficulty and time-consuming nature.

4.1.3 *Genetic tract tracing*

Transgenic expression of green fluorescent protein (GFP) and its spectral variants allows evaluation of axonal pathology and represented an important advance in neuroanatomical studies (Misgeld and Kerschensteiner, 2006). A group of mouse lines of considerable interest in neuroimaging are thy-1-XFP mice, which have been used to study the behaviour of specific neuronal tracts after spinal cord injury, including regenerative sprouting of dorsal column projections (Kerschensteiner et al., 2005), remodelling of CST components (Bareyre et al., 2005) and neuroprotection of corticospinal neurons after spinal injury (Carter et al., 2008). Analysis of labelled axons or cell bodies has been performed both using traditional post-mortem histochemical analysis (Bareyre et al., 2005; Carter et al., 2008) and *in vivo* time-lapse imaging

(Kerschensteiner et al., 2005). The use of these mice also avoids the methodological problems of tracer injection, such as invasive and technically demanding surgery, variability in labelling efficiency and local tissue damage at the site of injection (Kobbert et al., 2000). Important limitations of transgenic tract labelling include potential changes in expression pattern following injury and the bilateral nature of the labelling, preventing determination of the origin of newly grown fibres (e.g. Liu et al., 2010). In one such mouse, the YFP-H mouse, it has been reported that long-term expression of YFP accelerates age-related pathology, such as axonal swelling in certain CNS regions (Bridge et al., 2009). Therefore, results related to axonal pathology in affected CNS regions from older XFP-expressing mice must be interpreted carefully.

Viral vectors expressing fluorescent proteins represent a promising technique to label specific neuronal populations. They are versatile tools, as they can be injected unilaterally into an area of interest and label a specific neuronal population and, since they can encode fluorescent proteins, they also present the possibility of *in vivo* neuronal imaging. Adeno-associated viral (AAV) vectors are strongly neurotropic and have been shown to efficiently transduce neurons, producing impressive anatomical resolution of neurons and their branches, including long distance projections such as primary sensory neurons and the corticospinal tract (Towne et al., 2009; Mason et al., 2010; Blackmore et al., 2012; Hutson et al., 2012). Long-term gene expression may be achieved with AAV vectors, accompanied by only a minimal immune response (Chamberlin et al., 1998; McCown, 2005; Papale et al., 2009). Most AAVs use inverted terminal repeats (ITRs) from AAV2, but can be pseudotyped with the capsid from different serotypes of AAV, generating vectors with different cellular tropism and transduction properties (Burger et al., 2005; Vandenberghe et al., 2009). Recombinant AAV expressing the AAV1 or AAV5 capsid have been shown to have improved transduction profile and efficiency following injection into the mouse or rat brain

(Wang et al., 2003; Burger et al., 2004; Paterna et al., 2004). Drawbacks of viral vectors as tracers are similar to those of other injectable tracers, such as the possible damage caused by invasive injections and variability in the transduction efficiency between animals. This variability will not only be between individual animals according to injection technique, but will also be between batches of virus with different titres. The heterogeneity of serotype, promoter, envelope, transgene size and other factors also add to the complexity of this technique.

4.1.4 *Visualising plasticity*

The preceding sections have dealt with tracing techniques that visualise neurons in their entirety, either through cellular transport (e.g. HRP) or passive diffusion (e.g. GFP). These techniques are useful when examining the projection of a particular neuronal population, but do not provide evidence of synaptic connections. As discussed in previous chapters, several experimental therapeutic strategies lead to increased axonal growth, including regeneration of injured axons and sprouting of spared axons, as visualised using neuroanatomical tracers. Clearly, the formation of functional connections is required for these growing axons to mediate recovery but the anatomical evidence for this occurring is sparse. Several studies have attempted to show evidence of connectivity by combining tract tracing with electron microscopy (Alto et al., 2009; Liu et al., 2010) or immunostaining for synaptic proteins, such as synaptophysin (Houle et al., 2006; Liu et al., 2010; Rosenzweig et al., 2010).

The use of genetically encoded fluorescent probes to detect the morphological changes underlying synaptic plasticity has increased exponentially in recent years, vastly expanding scientific knowledge in the field of brain connectivity in health and disease (Tian and Looger, 2008). One group of these probes uses the pHluorin molecule

(Miesenbock et al., 1998), which is a pH-sensitive version of GFP. SynaptopHluorin (SpH) is the most commonly used pHluorin molecule and exploits the difference in pH between the lumen of a presynaptic vesicle and the extracellular fluid to report synaptic activity (Miesenbock et al., 1998; Burrone et al., 2006). Neurotransmitters are stored in presynaptic vesicles and released through exocytosis during synaptic transmission. The membrane containing vesicular proteins is subsequently re-internalised and reacidified during the process of endocytosis. By fusing the pHluorin molecule to the luminal end of the vesicular protein VAMP2, the pHluorin is exposed to a change in pH from ~5.6 to ~7.4 during exocytosis. In the acidic conditions of the vesicle, SpH fluorescence is quenched but when the pH is raised the fluorescence intensity increases, as illustrated in Fig. 4.1 (Burrone et al., 2006). In this way SpH can act as a sensor of presynaptic activity. This probe has been largely used *in vitro* to study presynaptic vesicle cycling (Li et al., 2005b).

4.1.5 *Aims of the chapter*

In this Chapter we combine the principles of neuroanatomical tract tracing with the use of a genetically encoded fluorescent probe that accumulates at presynaptic sites. First, the generation of lentiviral vectors encoding synaptic probes, and their use *in vitro*, are described. Application of lentiviral vectors to the adult rat brain led to suboptimal transduction *in vivo* and the application of AAV vectors to central and peripheral nerves was found to be superior. Finally, this Chapter describes the visualisation and quantification of synapses *in vivo* after application of an AAV vector expressing SpH to two classic neuronal projections: the CST and primary sensory neurons. In this way we describe the development of a novel tool for the visualisation of synapses *in vivo*.

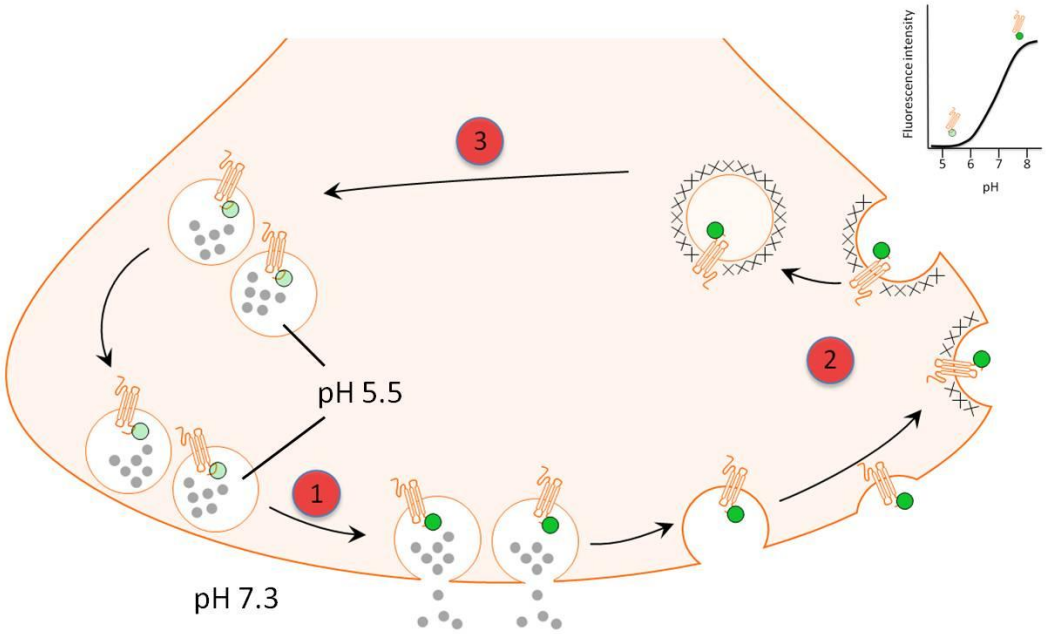


Figure 4.1: pH-dependent changes in synaptopHluorin fluorescence. Diagram illustrating the principle behind SpH as a marker of presynaptic activity. At rest, SpH resides inside synaptic vesicles where the intralumenal pH is acidic (pH ~5.5) and the fluorescence is quenched. Upon activation, exocytosis occurs (1), whereby vesicles fuse with the plasma membrane and the lumen is exposed to extracellular fluid, which is of neutral (pH ~7.3) and causes an increase in SpH fluorescence. Released vesicles are retrieved by endocytosis (2) and undergo reacidification an refilling with neurotransmitter (3). The return of the lumen to an acidic pH once again quenches the fluorescence. Adapted from Burrone et al., 2007.

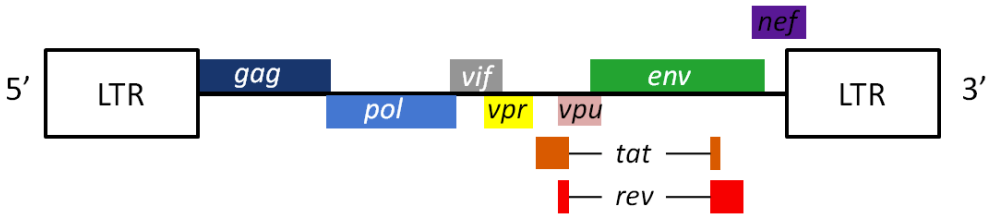
4.2 Materials and Methods

4.2.1 *Generation of lentiviral vectors*

Transfer plasmids. Two overexpression plasmids were generated, which expressed synaptopHluorin (SpH) or synaptophysin-GFP (Syn-GFP) under control of the cytomegalovirus (CMV) promoter. The original human immunodeficiency virus type 1 (HIV1) lentiviral transfer plasmid was assembled by Dr Ping Yip (King's College London, University of London) and contained a 5' central polypurine tract (cPPT) element and a 3' woodchuck post-tanscriptional regulatory element (WPRE) enhancer. The plasmid was altered by a commercial service (GeneScript) to include mCherry (Invitrogen) as reporter in place of GFP under the SFFV promoter (Fig. 4.2E). Dr Leon Lagnado (Cambridge University) kindly provided SpH and Syn-GFP cDNA plasmids. These two genes were subsequently cloned into the transfer plasmid backbone. The cDNA sequences were amplified by PCR with restriction sites at the end of the primers (XhoI/XbaI for SpH; XhoI/SpeI for Syn-GFP). The resulting products were then ligated into the lentiviral backbone. Successful insertion was ascertained by restriction enzyme digestion. Sequencing was also carried out for further confirmation (MWG Eurofins).

Packaging. A third generation lentivirus packaging system was used to package the newly generated vectors into virus particles, as previously described (Naldini et al., 1996; Dull et al., 1998; Yanez-Munoz et al., 2006). Briefly, the transfer plasmid was co-transfected with plasmids carrying essential viral genes (pMDLg/pRRE, pRSV.REV) and the viral envelope gene (VSV-G) into human embryonic kidney (HEK-293T) cells, using polyethylenimine (PEI) as a transfection reagent. All parent plasmids were the generous gift of Dr Rafael Yanez-Munoz (Royal Holloway, University of London) and are illustrated in Fig. 4.2. The transfection reaction was allowed to proceed for 4 hours at 37˚C before cells were washed and fed with complete

A HIV-1 provirus



B Packaging plasmid (pMDLg/pRRE)

C Rev plasmid (pRSV.REV)

D Envelope plasmid (pMD.VSV-G)

E Transfer plasmid (pRRL.CMV.SpH.SFFV.mCherry)

Figure 4.2: Plasmids used for the manufacture of lentiviral vectors. Schematic drawing of the HIV provirus and the four constructs used to make a lentivirus vector of the third generation. **A:** the proviral HIV-1 genome contains genes encoding proteins and sequences essential for the regulation of the virus life cycle. **B:** the packaging construct, pMDLg/pRRE, expresses the *gag* and *pol* genes under the CMV promoter. The *gag* and *pol* genes are expressed only when Rev promotes their nuclear export by binding to Rev-responsive element (RRE). **C:** The *rev* cDNA is expressed by a separate construct, pRRE.REV. **D:** pMD.VSV-G, encodes an envelope to pseudotype the vector, in this case VSV-G. **E:** The transfer plasmid, pRRL.CMV.insert.SFFV.mCherry, is the only portion transferred to target cells and contains the expression cassette for the transgene, SpH or Syn-GFP. The long-terminal repeats (LTRs) have been modified such that transduction results in a self-inactivating vector.

Dulbecco's modified Eagle's medium (DMEM; Invitrogen) daily while packaging occurred. The harvested HEK-293 cell medium was collected on days 2, 3 and 4 post-transfection and centrifuged at 690*g* for 10 minutes at room temperature and then filtered through a 0.22µm filter (Nalgene) to remove cell debris. The filtered medium was then harvested and transferred to high speed polyallomer centrifuge tubes (Beckman) and centrifuged at 50 000*g* in a SW32Ti rotor (Beckman) for 2 hours at 4˚C. The vectors were then resuspended in DMEM, centrifuged at 1400*g* for 10 minutes and incubated with $5Uml^{-1}$ DNaseI (Promega) and 10mM $MgCl_2$ (Sigma) for 20 minutes. The two vectors, LV-SpH and LV-Syn-GFP, were then aliquoted and stored at -80˚C.

Vector titration. An infection assay was used for lentiviral vector titration. For this, 10^5 HEK-293T cells were seeded on 6-well plated (Nunc). The following day, the cells of a spare well were collected by incubation with 0.25% Trypsin (Sigma) and the total cell number/well was calculated using a haemocytometer (Invitrogen). For titration, serial dilutions of each virus preparation ($1:10^3$, $1:10^4$, $1:10^5$ in 1ml) in DMEM media were supplemented with 16µg/ml Polybrene (Sigma) and added to the cells. After 72 hours, cells were fixed with 4% cold paraformaldehyde for 30 minutes and then washed 3 times in phosphate buffered saline (PBS) with 0.1% TritonX. Cells were immunostained for GFP by incubation with a rabbit anti-GFP antibody (1:1000; 60 minutes; Invitrogen), followed by three PBS washes and incubation with a donkey anti-rabbit Alexa Fluor 488 secondary antibody (1:1000; 60 minutes; Invitrogen) for 1 hour. Hoechst (1:5000; Sigma) was added to the secondary antibody solution to allow visualisation of nuclei. An IN Cell Analyser Workstation (GE Healthcare) was used to randomly acquire images from each well. Sample wells that contained <10% GFP-positive cells were used to determine the titre using the following formula:

transducing units (TU)/ml = (% eGFP positive cells/100) x cell number on day of transfection x dilution factor.

The titres we acquired for vector preparations varied from 10^6 to 10^8, with the highest titre preparations typically from day 2 of the harvest. For brain injections, vector from the highest titre preparation (1.27 x 10^8 TU/ml) was used.

4.2.2 *Application of lentiviral vectors in vitro*

Hippocampal culture. Primary hippocampal cultures were prepared from embryonic day 18 Wistar rat embryos of either sex. Hippocampi were excised from the brains of the embryos and dissociated using trypsin (5mg/ml for 15 minutes at 37˚C; Worthington) and plated at 350 cells/mm^2 on glass coverslips coated with poly-D-lysine (50µg/ml) and laminin (20µg/ml). Neurons were incubated at 37˚C and 5% CO_2 in Neurobasal media (company) supplemented with B27 (2%) and glutamax (500µM). Twice weekly 50% of the media was removed and replaced with fresh supplemented Neurobasal.

Lentiviral transduction. The number of biologically active virus particles per target cell is known as the multiplicity of infection (MOI). In these experiments lentiviral vectors were added at an MOI of 10, which typically produced close to 100% transduction of hippocampal neurons. Vectors were applied to the cultured cells 24 hours after plating and were left to express for 10-14 days: 10 days for immunocytochemistry and 14 days for SpH imaging.

Immunocytochemistry. After 10 days *in vitro*, hippocampal cells were washed with PBS and fixed for 30 minutes with ice cold 4% paraformaldehyde, then washed three times in PBS with 0.1% Triton (5 minutes each). Neurons were immunostained with mouse anti-βIII tubulin (1:1000; Promega) and rabbit anti-GFP (1:1000; Invitrogen) primary antibodies for 1 hour. After 3 further washes, as above, goat anti-mouse Alexa Fluor 546 antibody, donkey anti-rabbit Alex Fluor 488 antibody (both 1:1000; Invitrogen) and Hoechst (1:500; Invitrogen) were applied. Slides were coverslipped with Mowiol

mounting medium and images were captured on a confocal microscope (Carl Zeiss LSM 710).

SynaptopHluorin imaging. After 14 days *in vitro*, SpH imaging was performed using one of the hippocampal cultures, in the manner previously described (Fredj and Burrone, 2009; Burrone et al., 2007). Briefly, a coverslip is placed in a custom-built Perspex stimulation chamber (World Precision Instruments) containing two parallel platinum wires ~5mm apart, which depolarise cells when current is passed between them. Neurons were depolarised sequentially with 40, 100 and 300 stimuli at 20Hz using an SD9 stimulator (1ms, 25mA; Grass Instruments), whose timing was controlled by a TTL signal from the imaging software (Slidebook). Images were obtained using an inverted microscope Olympus IX71 with a CCD camera (Coolsnap HQ; Photometrics) controlled by Slidebook software (Intelligent Imaging Innovations). Images were analysed offline using ImageJ, using a region of interest (ROI) containing a high density of synapses (Fig. 4.3G). The change in fluorescence (ΔF) within the ROI in response to a stimulus was calculated by subtracting the initial fluorescence intensity (F_0) from the fluorescence of each time frame ($\Delta F = F - F_0$). A normalised value ($\Delta F/F$) is then calculated to account for differences in the number of reporter molecules in each ROI.

4.2.3 *Application of lentiviral vectors in vivo*

All procedures were carried out in accordance with UK Animals (Scientific Procedures) Act 1986 and approved by the local veterinarian and ethical committee. For all recovery surgery, sterile precautions were used. Adult male Wistar rats weighing 200-220g were anaesthetised with 60mg/kg ketamine and 0.25mg/kg medetomidine, diluted in saline and administered i.p. Body temperature was monitored rectally and used to regulate a homeothermic blanket. Four animals received intracortical brain injections of viral

vector (n=2 each of LV-SpH and LV-Syn-GFP). Rats were prepared for surgery by shaving and disinfecting the skin overlying the skull. The rat was placed into a stereotaxic frame (David Kopf Instruments) and the incisor bar adjusted such that Bregma and Lambda were at equal heights and the surface of the skull was flat. Following a skin incision along the midline, the skin and periosteum were retracted to expose Bregma. Six holes were drilled through the skull overlying the right sensorimotor cortex, according to coordinates obtained from microstimulation mapping studies (Neafsey et al., 1986). Coordinates are relative to Bregma, the midline and the surface of the brain and are defined as anterior-posterior (AP), medial-lateral (ML) and dorsal-ventral (DV), respectively:

1. 3.5mm ML; -0.5mm AP; 1mm DV
2. 3.5mm ML; +0.5mm AP; 1mm DV
3. 3.5mm ML; +2mm AP; 1mm DV
4. 2.5mm ML; +1.5mm AP; 1mm DV
5. 2.5mm ML; +0.5mm AP; 1mm DV
6. 1.5mm ML; +1.0mm AP; 1mm DV

6 injections of 0.5µl each were performed using a glass capillary pulled to a tip diameter of about 20µm, connected to a 10µl syringe and driven by a microdrive pump (Harvard Apparatus) at 0.25µl/min. The capillary was left in place for 2 minutes after the infusion to allow diffusion of the vector away from the injection site. Successful vector expulsion was confirmed visually. Overlying skin was sutured and 1mg/kg atipamezole hydrochloride was administered subcutaneously to reverse the anaesthetic. Animals recovered in an incubator and received 0.05mg/kg buprenorphine postoperatively. They were then left for 4 weeks to allow lentiviral transduction and expression of SpH and Syn-GFP.

4.2.4 *Application of adeno-associated viral vectors in vivo*

AAVs. Two AAV vectors were used in these experiments, both containing genomes from AAV2 pseudotyped with capsid serotype AAV5. The first was a commercially available vector, AAV2/5.CMV.hGHintron.GFP-Cre.SV40 (Cat # V1705, University of Pennsylvania Vector Core Facility), expressing a fusion protein of GFP and the enzyme Cre recombinase. This vector will hereafter be referred to as AAV-GFP-Cre. The titre of this preparation was nominally 2.0 x 10^{13} genomic copies per ml, although the Vector Core facility guaranteed a titre of only 2 x 10^{11}. The second vector, expressing SpH (AAV-SpH), was kindly provided by Professor R.M. Linden (King's College London). The titre of this preparation was 3.5 x 10^{12} genome copies per ml. The vectors contained transgene expression cassettes that carried the human cytomegalovirus (CMV) promoter to drive transgene expression, the transgenic cDNA and a downstream SV40 polyadenylation sequence. The transgene expression cassette is flanked by the non-coding ITRs, which are essential for packing of the viral vector DNA and are the only wild-type viral sequences in the vector. The AAV-SpH vector also contained a WPRE sequence downstream of these elements, which enhances the expression of the transgene (Peel and Klein, 2000).

Sciatic nerve injection. Animals were anaesthetised as above and the skin overlying the lateral aspect of the left hindlimb was shaved and disinfected. The sciatic nerve was exposed by blunt dissection between the musculus gluteus superficialis and musculus biceps femoris. A ~20µm tip diameter glass capillary connected to a microdrive pump (Harvard Apparatus) was used to deliver 5µl of AAV-GFP-Cre (n=6) or AAV-SpH (n=5) at 0.25µl/min. A subset of the AAV-GFP-Cre group of animals (n=3) received a distal sciatic nerve crush injury, via brief application of pressure with blunt forceps, immediately prior to nerve injection.

Intracortical injection. Rats were prepared for surgery, placed in a stereotaxic frame and 6 holes were drilled into the skull according to the coordinates listed in the previous section. In these experiments 6 0.5µl injections of AAV-GFP-Cre (n=3) or AAV-SpH (n=3) were performed using a 5µl syringe driven by a microdrive pump at 0.1µl/min. The needle was left in place for 10 minutes following each injection.

After all types of injection overlying muscles and skin were sutured and 1mg/kg atipamezole was administered s.c. Animals recovered in an incubator and received 0.05mg/kg buprenorphine postoperatively. All groups were left for 4 weeks to allow transduction and expression of GFP-Cre and SpH.

4.2.5 *Histology and immunohistochemistry*

Histology. Four weeks following AAV injection, animals were terminally anaesthetised with sodium pentobarbitone (80mg/kg, i.p.) and transcardially perfused with 200ml heparinised saline followed by 400ml paraformaldehyde (4% in 0.1M phosphate buffer). Brains, brainstems, spinal cords and dorsal root ganglia (DRGs) were dissected, as appropriate, from the animals in each group and post-fixed in 4% paraformaldehyde overnight at 4˚C. The following day the spinal cords and dorsal root ganglia were transferred into 20% sucrose for 48 hours at 4˚C and then blocked in OCT embedding compound (BDH) for cryostat sectioning. 20µm thick DRG sections and sagittal spinal cord sections were cryostat cut and thaw-mounted onto Superfrost Plus slides and stored at -20˚C. Brains and brainstems were embedded in 10% gelatin (Sigma) and placed in 4% paraflormaldehyde overnight to harden. They were cut rostro-caudally into 30µm coronal serial sections using a Vibratome (VT 10005; Leica) and collected into a 24-well plate (Nunc) containing PBS and 0.1% azide (Sigma).

Immunohistochemistry. Brain and brainstem tissue was stained using the free-floating method; spinal cords and DRGs were stained on microscope slides. Tissue sections

were washed 3 times for 5 minutes each in PBS and immunostained with a rabbit anti-GFP antibody (1:1000, Invitrogen), diluted in PBS with 0.2% Triton and 0.1% azide, overnight at room temperature. Primary antibody was then removed, sections were washed 3 times in PBS and incubated with a goat anti-rabbit Alexa Fluor 488 secondary antibody (1:1000, Invitrogen) for 2 hours at room temperature. Finally sections were washed 3 times in PBS, and mounted if necessary (brain and brainstem sections), and coverslipped using Vectashield with DAPI (Vector Labs). DRG sections were double stained with a mouse anti-β3 tubulin primary antibody and donkey anti-mouse Alexa Fluor 546 secondary antibody.

4.2.6 *Imaging and quantification*

For quantification of the number of transduced neurons in DRGs after sciatic nerve or spinal cord injection of AAV, low power mosaic images were taken on an Apotome microscope (Carl Zeiss) using the x 20 objective. Using Image J (NIH), we counted the total number of β3 tubulin labelled profiles and the number of GFP labelled profiles in 4 sections per DRG for each animal and used these as an approximation of the percentage of transduced neurons ((no. GFP-positive profiles / no. β3 tubulin-positive profiles) x 100).

For quantification of the number of transduced CST fibres at the C2 level of the spinal cord following brain injection of AAV, high resolution mosaic Z-stack images of transverse cord sections were captured on a confocal microscope (Carl Zeiss LSM 710) using a x 40 objective. The maximum intensity projection (MIP) from this stack was used to determine the number of fibres in the dCST by performing manual counts using Image J (NIH).

The number of presynaptic boutons labelled by the virus was determined in transverse sections of the cervical cord of brain-injected animals and in sagittal sections of the

lumbar cord of sciatic nerve-injected animals. Three sections per animal were analysed. High resolution, mosaic images were captured, with Z-stacks including only the middle 10µm of the section, such that the top and bottom of the section are discarded. Images were converted to MIPs and saved in an .LSM format before analysis using Image J (NIH) was performed. The area imaged included all GFP-positivity in the grey matter of the spinal cord (i.e. excluding the dCST). Initially, the 'Despeckle' function was used to remove noise from the image and a threshold was applied to achieve a binary image. An automated counting function ('Analyse Particles') then determined the number of synaptic boutons. Only objects that had a diameter greater than 0.4µm were included in the analysis, to eliminate small, non-specific GFP-positive objects. To establish the threshold chosen for each image, 5 sample areas per section were chosen and manual counts of boutons were performed. A range of thresholds was applied and the number of particles detected by the program was compared to the manual count. In this way, an optimal threshold was chosen and applied to the entire image (Fig. 4.9).

4.3 Results

4.3.1 *LV-SpH and LV-Syn-GFP allow visualisation of synaptic puncta in vitro*

To assess whether the lentiviral vectors generated (LV-SpH and LV-Syn-GFP) had been successfully packaged they were applied to dissociated hippocampal neurons from embryonic rats. This well characterised experimental system has been widely used for the study of synaptogenesis and synaptic plasticity *in vitro* (Verderio et al., 1999). Lentiviral vectors were added to culture medium after 24 hours *in vitro* and 10 days later neurons were fixed and stained for GFP and the neuronal marker β3 tubulin (Fig. 4.3A-F). This time point was chosen as 7-9 days *in vitro* is a period of peak synaptogenesis in cultured hippocampal neurons (Fletcher et al., 1991). Indeed, punctate GFP staining was observed on the cell bodies and neurites of the cultured neurons transduced with either LV-Syn-GFP (n=5; Fig. 4.3A-C) or LV-SpH (n=5; Fig. 4.3D-F). Using LV-SpH and LV-Syn-GFP at an MOI of 10 led to neuronal transduction of close to 100%. These findings indicate that LV-Syn-GFP and LV-SpH successfully transduce cultured hippocampal neurons and appropriately express synaptic proteins tagged with GFP.

4.3.2 *SynaptopHluorin delivered by lentiviral vector can be used to detect exocytosis*

We next tested whether the lentivirally expressed SpH could detect the pH change that occurs during exocytosis. To do this, live neurons were inspected under an inverted fluorescent microscope and a field of view with a dense concentration of synapses was chosen (Fig. 4.3G). The response of these puncta was evaluated in response to electrical stimulation with incremental numbers of stimuli (40, 100 and 300 stimuli at 20Hz). At rest, synapses emitted a low level of fluorescence, which increased rapidly during the electrical stimulation and subsided more gradually during the recovery phase (Fig.

4.3G). As expected, quantification of the change in fluorescence, normalised to the resting fluorescent intensity, showed that the increase in fluorescence correlates with the number of action potentials used to stimulate the culture (Fig. 4.3H). These findings indicate that LV-SpH can transduce hippocampal neurons in culture, the SpH construct can be targeted to presynaptic locations and respond appropriately to stimulation. Thus, LV-SpH can be used to monitor synaptic function in cultured neurons. Because the *in vivo* applications of this vector showed little sign of widespread neuronal labelling (see next section), these pilot *in vitro* observations were not pursued.

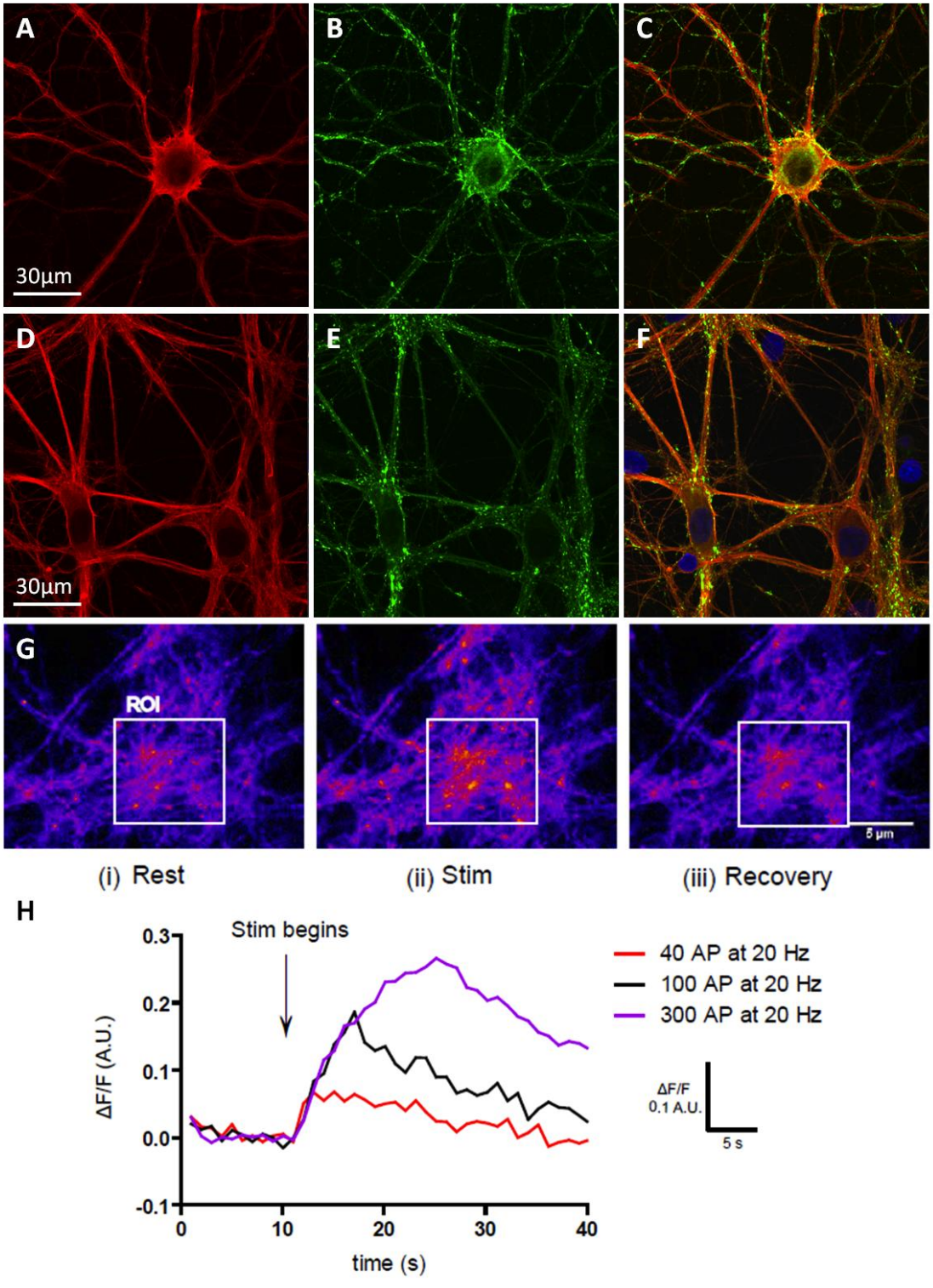

A
B
C
30µm
D
E
F
30µm
G
ROI
5 µm
(i) Rest
(ii) Stim
(iii) Recovery
H
Stim begins
ΔF/F (A.U.)
0.3
0.2
0.1
0.0
-0.1
0
10
20
30
40
time (s)
40 AP at 20 Hz
100 AP at 20 Hz
300 AP at 20 Hz
ΔF/F
0.1 A.U.
5 s

Figure 4.3: *In vitro* application of lentiviral vectors expressing SpH or Syn-GFP. A-F: dissociated embryonic rat hippocampal neurons immunostained for β-III tubulin (red) and GFP (green) after 10 days *in vitro* and incubation with LV-Syn-GFP (**A-C**) or LV-SpH (**D-H**). **G-H**: SpH responses to electrical stimulation of cultured hippocampal neurons transduced with LV-SpH. **G**: Typical response from axons expressing SpH to a train of high-frequency stimuli. Representative images are selected for display from the time series: (i) pre-stimulation (rest), (ii) peak of response (stim) and (iii) post-stimulation (recovery). A region of interest (ROI) encompassing an area with a high density of SpH-expressing synapses was chosen. **H**: changes in SpH fluorescence (ΔF/F) of neurons when stimulated with 40, 100 or 300 action potentials at 20Hz. Synapses show increases in fluorescence during the stimulus (exocytosis) and a more gradual recovery following the end of the stimulus (endocytosis and reacidification).

4.3.3 *Brain injection of LV-SpH leads to limited neuronal transduction*

Having shown that LV-SpH can be used to visualise synapses and detect synaptic function in cultured neurons, we next tested this vector *in vivo*. LV-SpH was injected unilaterally into the sensorimotor cortex and 4 weeks later brains were examined for GFP expression. A small, localised area of GFP-positivity was observed in the cortex of injected rats (n=3; Fig. 4.4A). Higher magnification analysis of the transduced cells revealed the presence of GFP-positive cells of both neuronal and glial appearance (Fig. 4.4B). Sensorimotor cortex injections of viral vectors have been used in a previous study and have achieved efficient CST transduction, with GFP-positive axonal projections observed in the brainstem and spinal cord (Blackmore et al., 2012). In our experiments we did not observe any transduced axons at or below the level of the brainstem (data not shown). Thus, LV-SpH, at least as used here, leads to suboptimal neuronal transduction after brain injection.

4.3.4 *Analysis of AAV2/5 vector ability to transduce neurons in vivo*

Since *in vivo* injection of a lentiviral vector led to suboptimal transduction, a pilot experiment was carried out using an AAV vector available in our laboratory, AAV-GFP-Cre. This vector expresses the enzyme Cre recombinase, with a GFP tag. In this pilot experiment we did not use the enzymatic property of the Cre but were rather interested in the ability of an AAV2/5 vector to transduce neuronal populations, compared to a lentiviral vector. The viral vector was injected into different neural tissues of adult rats at the titre supplied (2×10^{13}) to examine the transduction of neuronal populations, including (i) brain injections, targeting CST neurons (n=3), (ii) spinal cord injections, targeting spinal neurons (n=3), and (iii) sciatic nerve injections, targeting sensory neurons (n=6). Four weeks after injection into the sensorimotor cortex, a substantial cortical area was found to be GFP-positive and transduced axons were

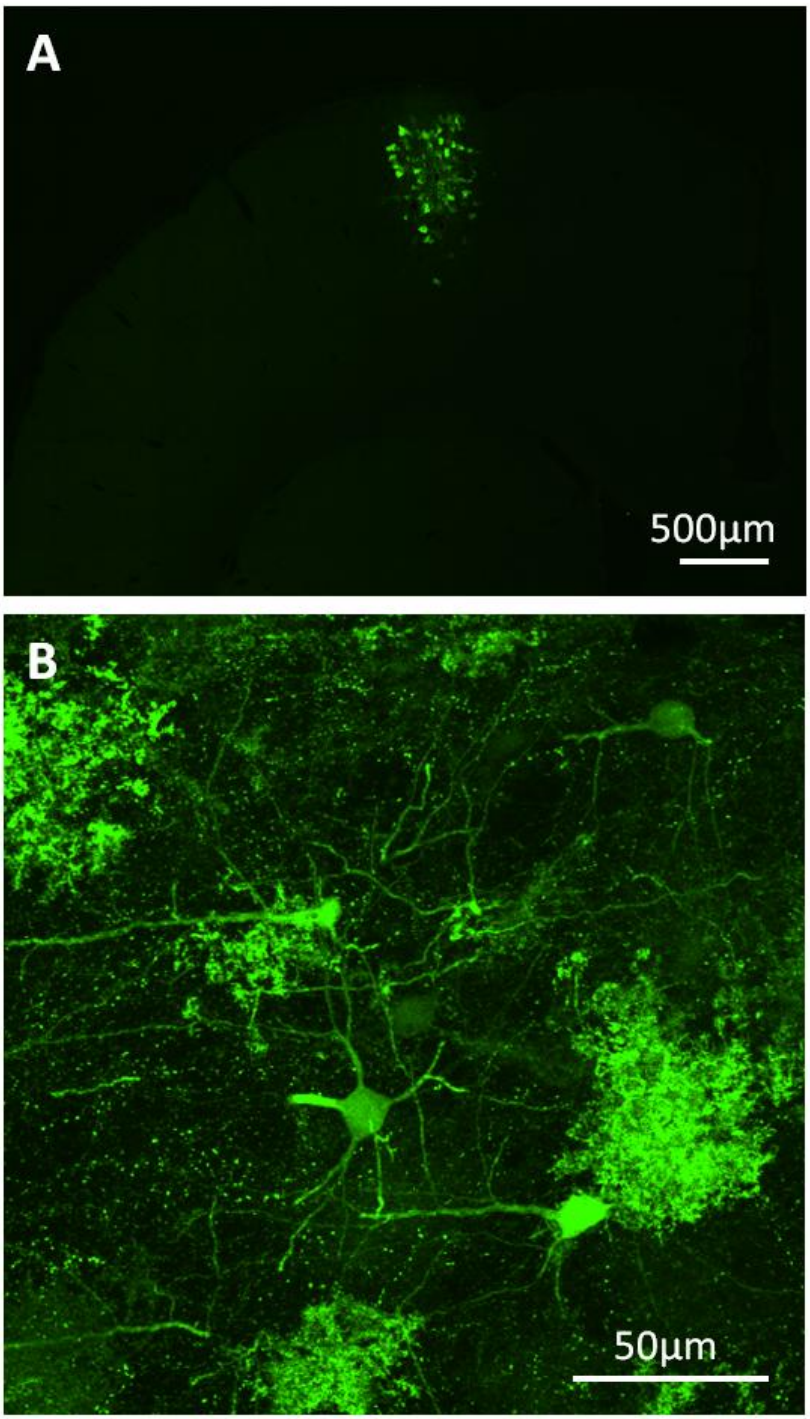


Figure 4.4: *In vivo* application of lentiviral vectors. A: adult rat brain at one month after injection of LV-SpH into the somatosensory cortex, immunostained for GFP. A small area of GFP reactivity is present in a localised area within the cortex. **B**: high power image of transduced cells. Both neuronal and glial cells are transduced by the vector.

observed to have projected away from the injection site and into thalamic areas (Fig. 4.5A). Higher magnification imaging revealed transduced cells to be neuronal in appearance (Fig. 4.5B).

Spinal cord injection of AAV-GFP-Cre similarly led to transduction of spinal neurons in the grey matter of the spinal cord, iplsilateral to the injection (n=3; Fig. 4.5C). Interestingly, GFP-positive neurons were also observed in local DRGs following spinal cord injection (Fig.4.5D), indicating retrograde transport and transduction of sensory neurons after spinal injection. Quantification of the number of transduced sensory neurons in cervical DRGs following spinal cord injection (Fig. 4.6A) showed that the highest number of GFP-positive cells was found in the DRG immediately adjacent to the injected spinal segment, C5 (19.9±7.1%), followed by the rostral C4 DRG (15.2±2.5%). A smaller number of GFP-positive neurons were detected in the DRG immediately caudal to the injection site, C6 (5.5±0.03%).

To investigate the ability of an AAV2/5 vector to transduce sensory neurons after distant injection into the sciatic nerve, we examined GFP expression in lumbar DRGs 4 weeks after sciatic nerve injection of AAV-GFP-Cre. We also investigated whether simultaneous nerve crush could improve the transduction efficiency after AAV injection. When injected into the intact sciatic nerve, AAV-GFP-Cre transduced a small number of DRGs (Fig. 4.5E). Most positive cells had GFP immunolabelling only in the nucleus (Fig. 4.5F), presumably due to the nuclear targeting of the Cre part of the Cre-GFP fusion protein. Occasional cells with diffuse GFP staining throughout the cytoplasm were observed, likely due to transduction of that particular neuron by a greater number of viral particles. Quantification of the number of GFP-positive profiles revealed that the highest numbers of transduced cells were in the L4 DRG (L4: 2.8±2.2%, L5: 1.6±1.3%, L6: 1.43±0.6%, n=3; Fig. 4.6B). Subjecting the sciatic nerve to a crush injury at the time of nerve injection led to a significant increase in the

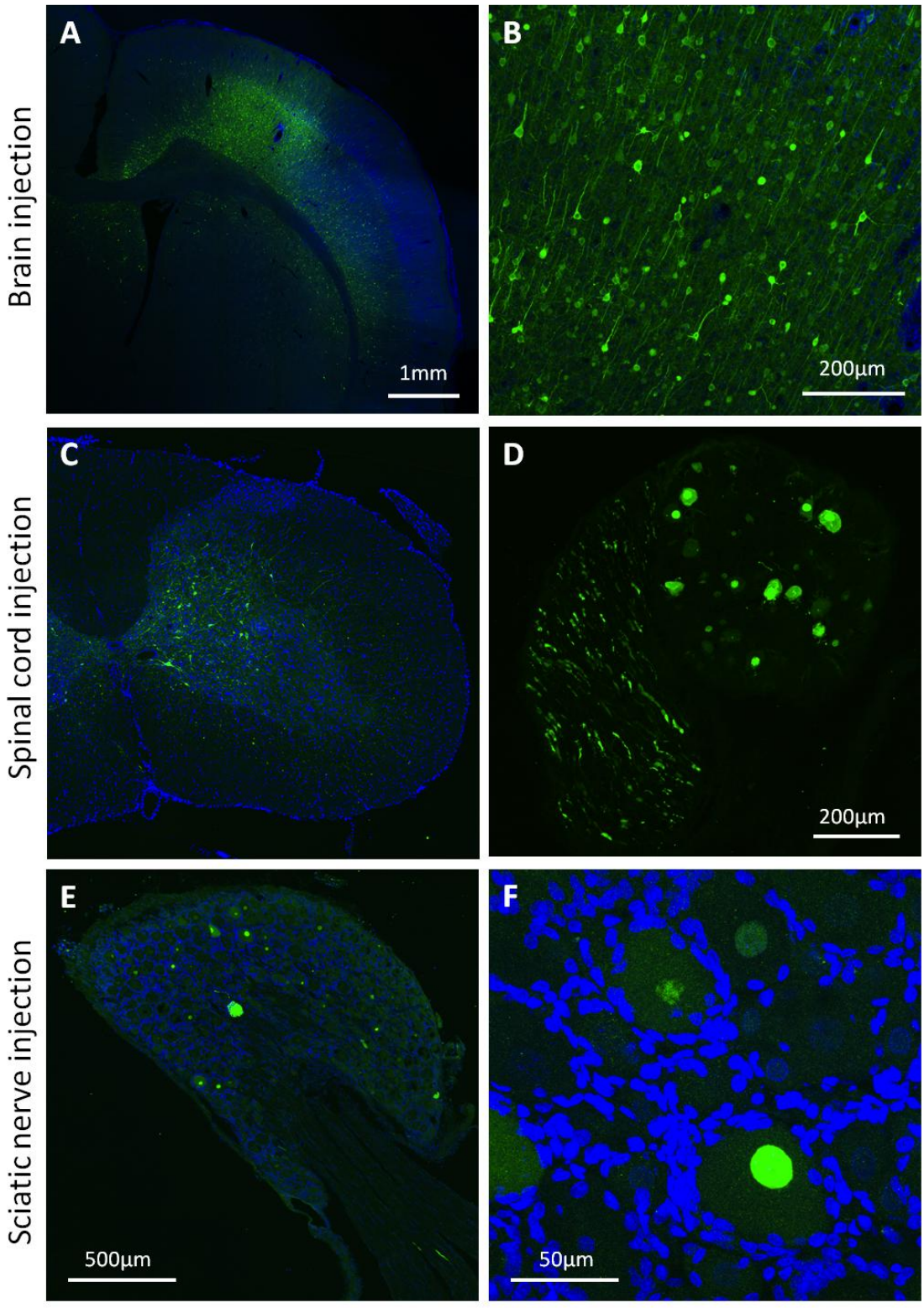
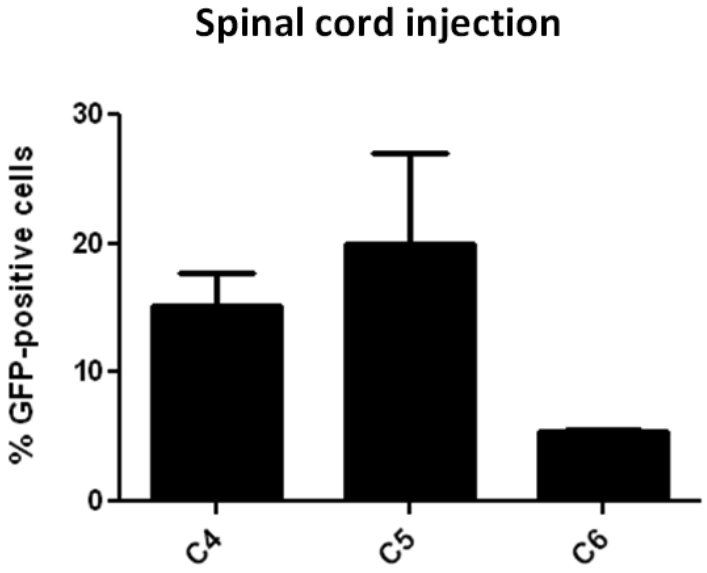

Brain injection
Spinal cord injection
Sciatic nerve injection
A
B
C
D
E
F
1mm
200µm
200µm
500µm
50µm

Figure 4.5 ***In vivo*** **application of AAV2/5.** AAV-GFP-Cre was applied to three nervous system locations: brain (**A**, **B**), spinal cord (**C**, **D**) and sciatic nevre (**E**, **F**). Sections were immunostained for GFP (green) and DAPI (blue). Low power images of each tissue are shown in the left panels (**A**, **C**, **E**). **A:** coronal section of AAV-injected brain, showing a large area of transduction with GFP visible in the thalamus as well as in the cortex. **B**: high magnification image of the cortical GFP-positive area in **A**, showing that all visible cells have a neuronal morphology. **C:** transverse section of AAV-injected cervical spinal cord, showing numerous transduced GFP-positive cells in the grey matter of the spinal cord. **D**: C4 DRG from a spinal cord injected animal containing retrogradely transduced cells. GFP-positive axons are also visible running in the dorsal root**. E:** L4 DRG from a sciatic nerve injected animal that received a nerve crush at the time of injection, showing ~8% transduction of DRG neurons. **F**: High magnification image of the lumbar DRG in **E**, showing three GFP-positive nuclei.

A


Spinal cord injection
% GFP-positive cells
30
20
10
0
C4
C5
C6

B

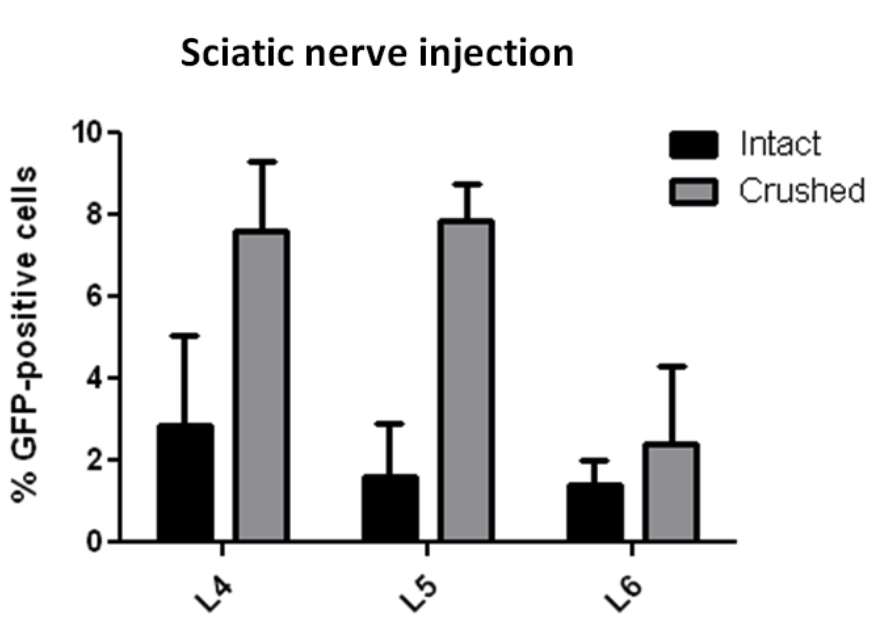

Sciatic nerve injection
% GFP-positive cells
10
8
6
4
2
0
L4
L5
L6
Intact
Crushed

Figure 4.6 Quantification of GFP-positive profiles in DRGs following AAV2/5 injection. A: Spinal cord injection of AAV-GFP-Cre led to retrograde transduction of sensory neurons in cervical DRGs adjacent to the injection site (n=3). The highest number of transduced cells was in the C5 DRG, immediately adjacent to the injection site in the C5 segment of spinal cord. Positive cells were also observed in the DRGs immediately rostral and caudal to the injection, C4 and C6. **B**: Sciatic nerve injection of AAV-GFP-Cre led to retrograde transduction of DRG neurons. Injection into the intact nerve led to the transduction of a modest number of cells. Performing a nerve crush at the time of injection led to a significant increase in the efficiency of retrograde transduction. Data are shown as mean ± SEM.

number of transduced neurons in lumbar DRGs (L4: 7.6±1.7%, L5: 7.8±0.9%, L6: 2.4±1.9%; n=3, significant effect of nerve crush, $p<0.05$, unpaired t-test on L4-6 pooled for crushed and intact groups). This pilot experiment shows that AAV2/5 is capable of transducing large numbers of brain and spinal cord neurons when directly injected into the vicinity of their cell bodies, and can transduce a modest number of sensory neurons in a retrograde manner when injected into the sciatic nerve. Based on their promising transduction profiles we decided to use AAV2/5 vectors for further *in vivo* experiments.

4.3.5 *CST transduction using a synaptopHluorin-expressing AAV2/5 vector*

To investigate the synapses made by CST projections, we used an AAV vector expressing a GFP-tagged protein that accumulates at presynaptic sites, AAV-SpH, to transduce corticospinal neurons. This vector was made in the laboratory of Professor Michael Linden (King's College London). We injected the vector unilaterally into the sensorimotor cortex of adult rats (n=3). Four weeks post-injection intense GFP expression was visible at the injection site (Fig. 4.7A) and many labelled neurons were also visible further away, extending throughout the ipsilateral cortex (Fig. 4.7B). Higher magnification imaging revealed that the majority of labelled cells had a neuronal morphology (Fig. 4.7C).

To specifically assess CST transduction, the brainstems of brain-injected animals were immunostained for GFP in order to visualise SpH. Many transduced, SpH-positive axons were visible in the right medullary pyramid (Fig. 4.8A, B), where the tract from the right cortex is located as it projects towards the spinal cord. Below the level of the pyramids, at the spinomedullary junction, the CST decussates and projects dorsally, where it resides mainly in the dorsal columns of the spinal cord. GFP-positive axons transduced by AAV-SpH were visible in the left dorsal column (Fig. 4.8C, D). The mean number of transduced axons at the cervical level was 2701±436 (n=3).

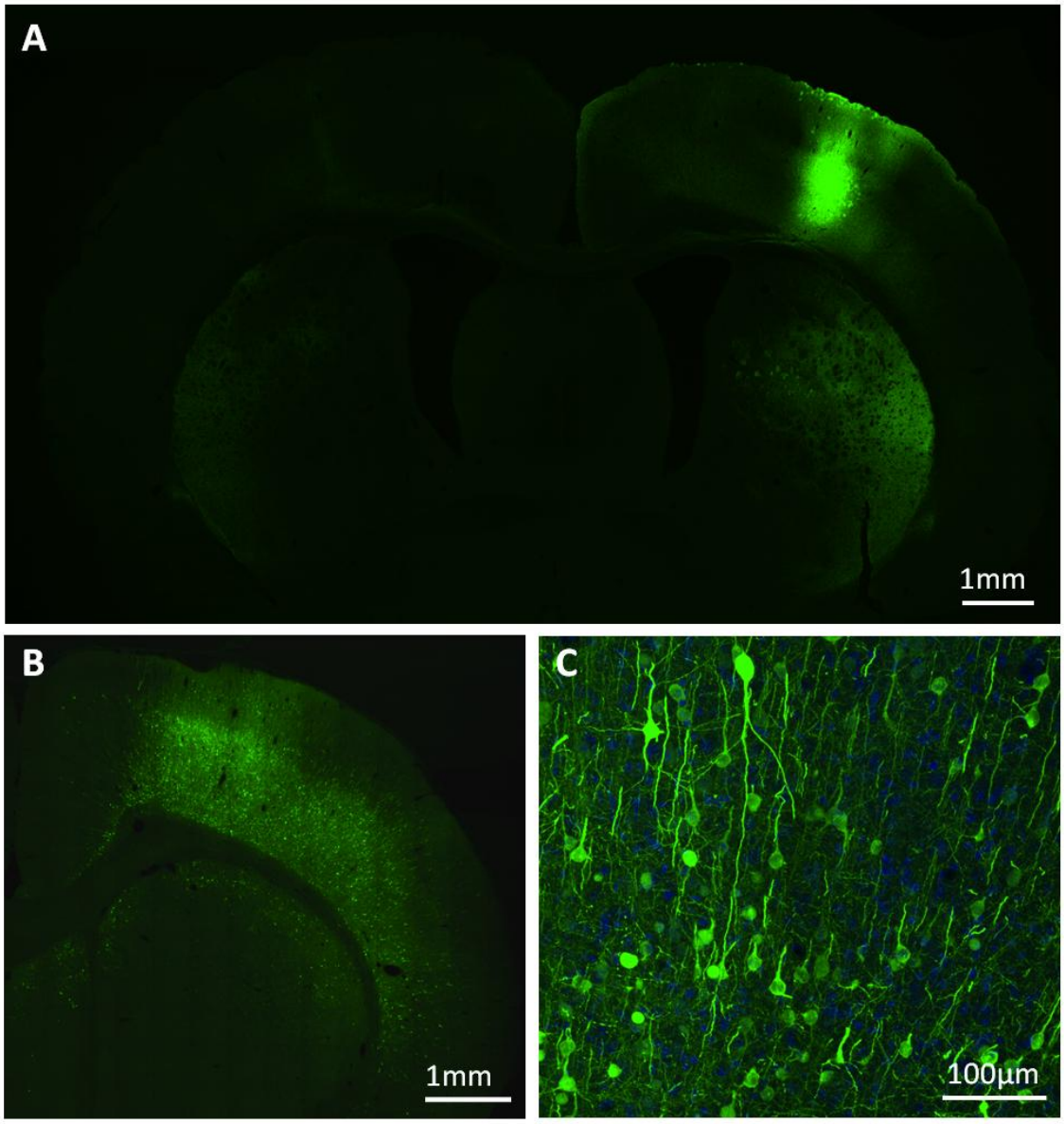
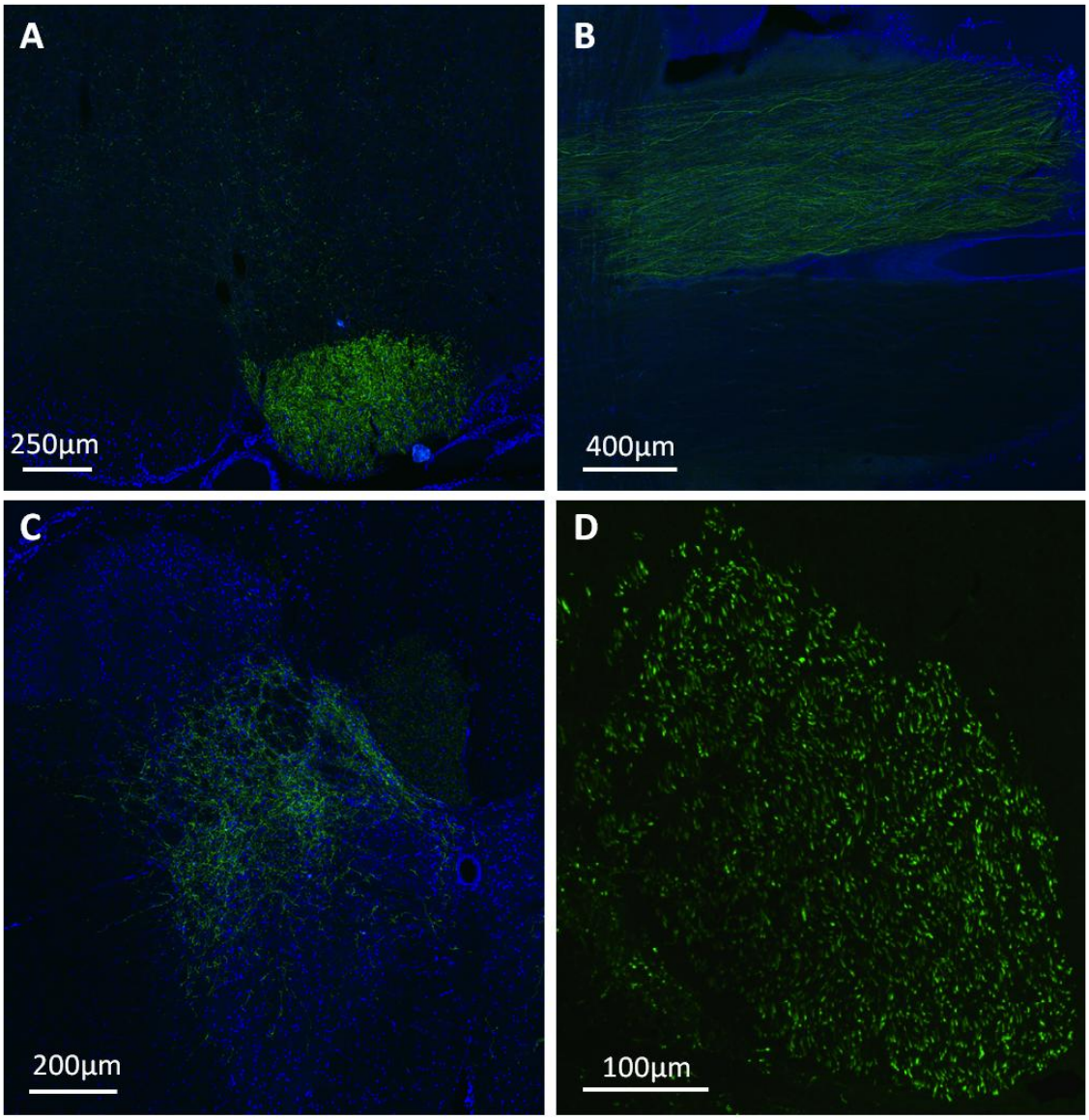

A
1mm
B
1mm
C
100µm

Figure 4.7: Brain transduction following cortical injection of AAV-SpH. A: Representative images showing expression of SpH in the brain four weeks after AAV-SpH injection. Bright SpH labelling is observed at the injection site and in the ipsilateral thalamus. A small area of contralateral thalamic labelling is also present. **B**: Image from a different brain section in the same animal showing a widespread area of transduction extending throughout the cortex. **C**: High power image of an SpH-positive area in **B**, showing SpH present throughout the cytoplasm of the transduced cells, which have a mostly neuronal morphology.


A
250µm
B
400µm
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200µm
D
100µm

Figure 4.8: SpH is apparent throughout the CST projection to the brainstem and spinal cord. Sections were immunostained for GFP (green) and DAPI (blue). **A**: coronal brainstem section showing SpH-positive axons in the pyramid ipsilateral to AAV-SpH injection. **B**: horizontal brainstem section showing the same tract longitudinally. Again, the ipsilateral tract contains many SpH positive axons. **C**: transverse section of spinal cord at the C2 level. CST axons can be seen leaving the white matter tract and terminating in the grey matter of the spinal cord. The terminal branches are found in the intermediate laminae of the spinal grey matter, but a small number extend towards the ventral horn. **D**: higher magnification image showing the dorsal CST at the C2 level of the spinal cord. A large number of transduced CST axons are present in the tract at this level.

4.3.6 *AAV-SpH labels synapses made by CST neurons in cervical spinal cord*

Axons leave the CST throughout the spinal cord to make connections with neurons in the grey matter of the spinal cord. This phenomenon was observed at the cervical level of brain-injected animals, where dense SpH labelling is observed in the intermediate laminae of the spinal grey matter (Fig. 4.8C). Although the CST projections appear axonally labelled, when examined at higher power SpH was found to accumulate in large numbers of bright puncta resembling presynaptic boutons (Fig. 4.9A, B; 4.10A). These will henceforth be referred to as synapses, although what else they might also represent is discussed later. To quantify the number of synapses, we took high-resolution images of the ipsilateral and contralateral spinal grey matter. During offline analysis, a threshold was chosen for each section and applied to create a binary image. Fig. 4.9 describes the method of thresholding, which involves performing manual counts on sample regions from the grey matter and choosing the optimum threshold to be applied to the whole image. The number of objects recognised after creation of the binary image was determined using an automated counting function in the imaging software. Any object with a diameter of less than 0.4μm was excluded from analysis to prevent the inclusion of small, non-specific areas of fluorescence. The spinal cords from 3 animals were imaged and SpH-positive synapses were counted in several sections. As expected, a greater number of synapses were detected in the grey matter of the animal with the largest number of SpH-positive dorsal CST axons and the smallest number in those animals with the fewest transduced CST neurons (Fig. 4.10C). There was very little variability between different sections from the same animal, as shown by the error bars in Fig. 4.10C.

These findings indicate that AAV-SpH can be used to transduce corticospinal neurons and visualise their presynaptic boutons in the spinal cord in a quantifiable way.

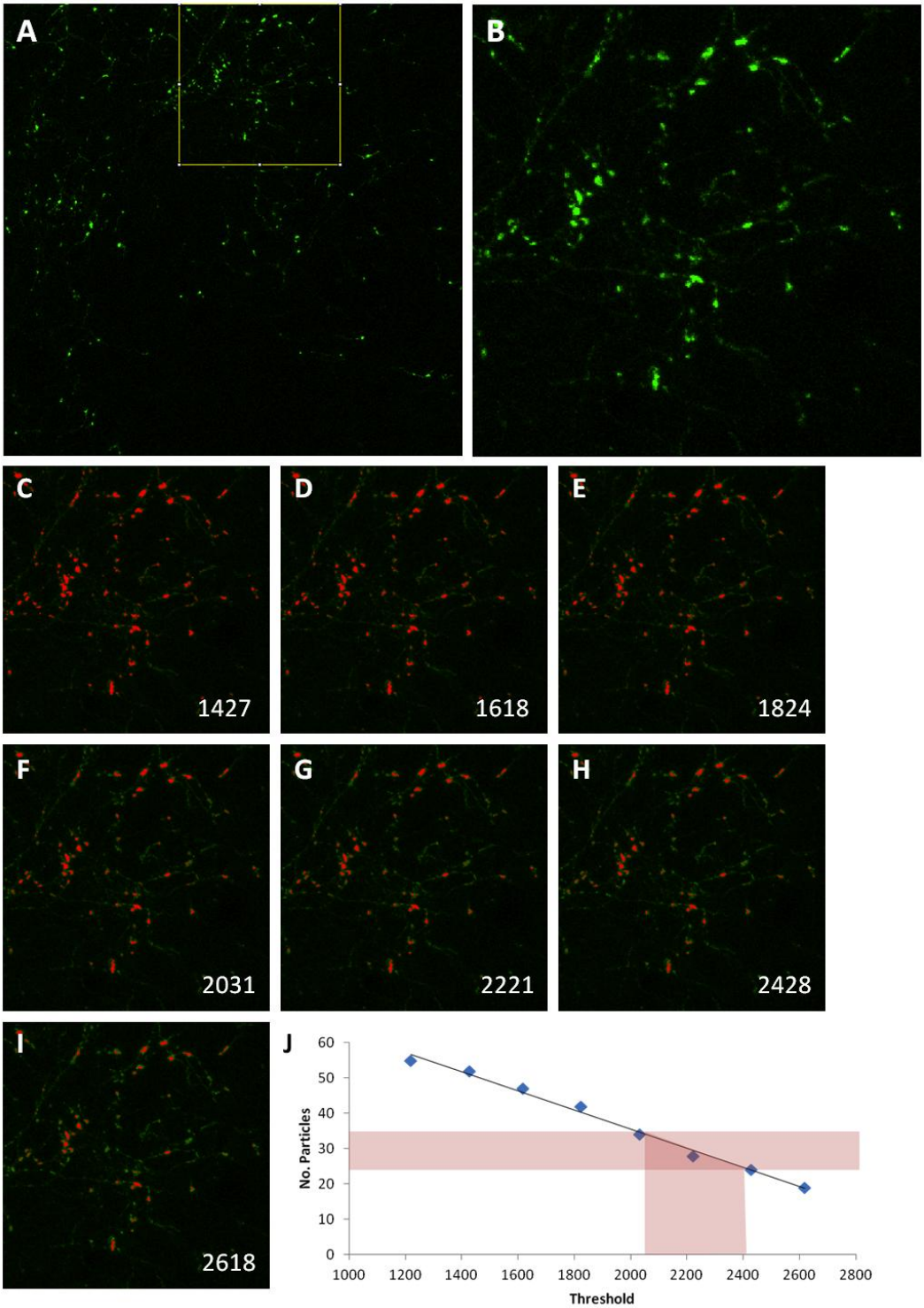
A
B
C
1427
D
1618
E
1824
F
2031
G
2221
H
2428
I
2618
J
No. Particles
60
50
40
30
20
10
0
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
Threshold

Figure 4.9: Determination of threshold level for quantification of synapse number. A: Example of one tile of a large mosaic of maximum intensity images from a 10µm thick Z stack. The yellow box indicates the 360 x 360µm area used for threshold determination. **B:** Enlargement of the boxed area in A. One of five 360 x 360µm areas chosen from throughout the grey matter of a given spinal cord section. A manual count of the maximum and minimum possible number of boutons in the image is performed. **C-I:** A series of thresholds are applied to the image and the number of objects is calculated using a counting function of ImageJ. **J:** The number of objects detected is plotted against the threshold applied to the image. A lower threshold allows a greater number of objects to be recognised. Using this range and the ranges from the four other sample areas throughout the section, an optimum threshold is chosen and applied to the entire image. An automated counting protocol is used to avoid bias in the counting process and because of the large number of synapses within each spinal cord section.

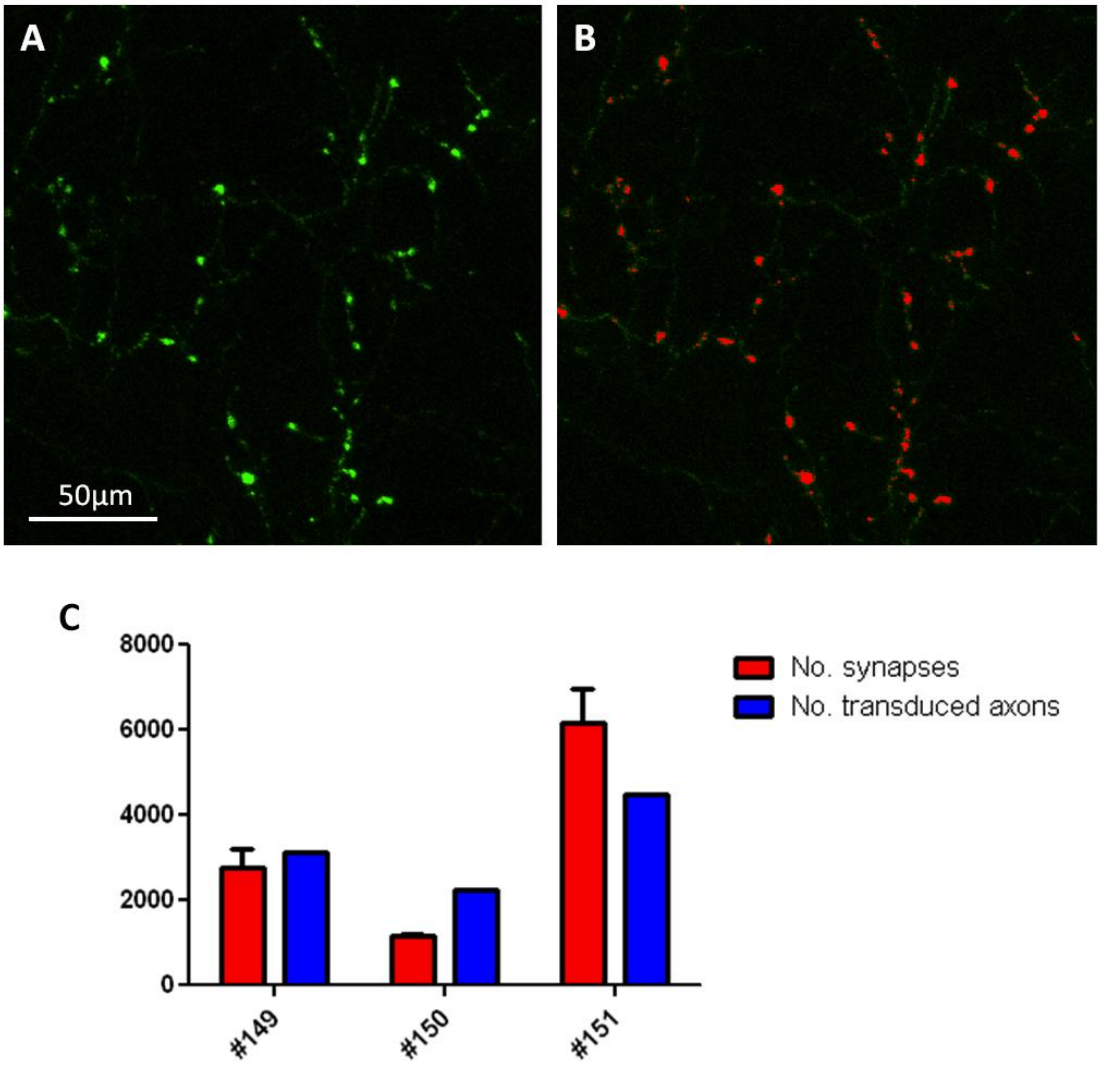
A
B
50µm
C
8000
6000
4000
2000
0
No. synapses
No. transduced axons
#149
#150
#151

Figure 4.10: Quantification of the number of synapses in C2 spinal cord following brain injection of AAV-SpH. A: At high magnification the terminal branches of CST axons are faintly labelled with many bright SpH-positive puncta along their length, presumably presynaptic specialisations. **B**: Example image illustrating how quantification of the numbers of synapses is carried out. First non-specific noise is removed from the image using the 'Despeckle' function in ImageJ. A threshold is then applied, which creates a binary image. Any recognised object is then counted, excluding any object smaller than 0.4μm. This process is applied to the entire grey matter of each spinal cord section analysed. **C**: Data are shown separately for the three animals that received brain injections. Quantification of the number of transduced CST axons (blue bars) at the C2 level was performed by a manual count of the number of axons in the dorsal column projection in a section of spinal cord from this level. Quantification of the number of synapses (red bars) is shown for each animal. The animal with the greatest number of transduced axons also had the greatest number of synapses and vice versa. To determine the consistency of the number of synapses between sections, three sections from the cervical cord were analysed for each animal and the data are shown as the average number of synapses in these three sections. Error bars (SEM) are small, indicating that the variability between sections is low.

4.3.7 *Sensory neuron synapses can be visualised using AAV-SpH*

We next investigated whether the synapses made by a second population of neurons, primary afferent sensory neurons, could be visualised using AAV-SpH. For this we injected AAV-SpH into the sciatic nerves of adult rats and examined SpH expression in the lumbar spinal cord (n=5). Four weeks following viral vector injection, SpH was expressed within the dorsal portion of sagittal sections of lumbar spinal cord within branching afferent fibres (Fig. 4.11A) of two rats. Unfortunately, as this work is still underway the remaining three rats have not yet been characterised, so the results reported here will refer to the two animals with complete datasets. Primary afferent terminals in the spinal dorsal horn of the analysed spinal cords were visible as bright SpH-positive puncta (Fig. 4.11C). Quantification of the number of labelled synapses in three sagittal spinal cord sections was performed as described above. Three sections were analysed for each animal using the method described above for CST terminals. High resolution images of all areas of SpH-positivity were taken and converted to binary images, prior to automated object counting. The total number of synapses counted in this way was 809 for animal #1, and 1504 for animal #2. To account for differences in the number of neurons transduced by AAV-SpH, it is necessary to normalise the number of SpH labelled synapses in the spinal cord to the number of transduced sensory neurons. Sciatic nerve injection of AAV-SpH into intact nerves led to transduction of a small number of DRG neurons (1.2% at L4, n=3; Fig. 4.11B), comparable to AAV-GFP-Cre injection into intact nerves described above. It will be possible to get a better idea of the usefulness of AAV-SpH as a tool for visualising primary afferent terminals when the spinal cords of all rats in this group have been fully analysed.

These findings indicate that it is possible to visualise the synaptic connections of sensory neurons transduced with AAV-SpH. Despite the modest number of transduced

sensory neurons, it was possible to visualise individual synapses in the spinal cord in a quantifiable way.

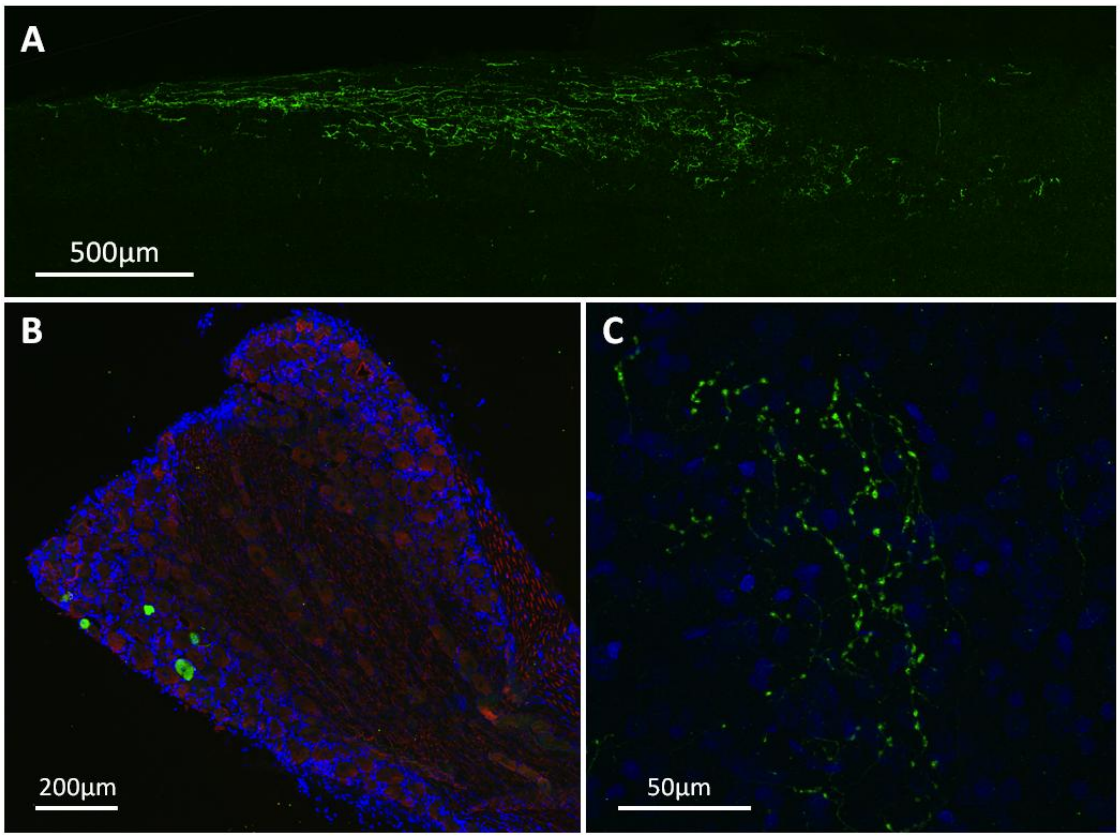

Figure 4.11 Spinal cord and DRG expression of SpH following sciatic nerve injection of AAV-SpH. A: Sagittal section of spinal cord showing branches of primary afferent neurons expressing SpH in the dorsal grey matter of the spinal cord. **B**: Small numbers of SpH-positive DRG neurons (green) in an L4 DRG also immunostained for β3 tubulin (red) and DAPI (blue). **C**: High magnification of SpH-positive primary afferent terminals showing presynaptic specialisations as varicosities along the terminals.

4.4 Discussion

This Chapter has described the development of a novel tool that allows visualisation of synapses. Two neuronal populations, corticospinal neurons and primary afferent sensory neurons, were investigated in the adult rat. An AAV2/5 vector, AAV-SpH, was found to efficiently transduce corticospinal neurons and label synapses distant from the site of vector injection in the cortex via anterograde transport of SpH. Similarly, AAV-SpH transduced a modest number of sensory neurons in a retrograde fashion after peripheral nerve injection and labelled their central terminals. Thus, AAV-SpH has been shown to label the synapses of both peripheral and central nervous system neurons and presents the potential for an anatomical measure of synaptogenesis.

4.4.1 *Using viral vectors to transduce the CST*

AAV vectors are commonly used for gene delivery to the CNS because they permit non-toxic transduction of neurons, resulting in long-term gene expression (Bartel et al., 2012). These features make AAV vectors attractive candidates for translation into the clinic. Indeed, a number of trials involving gene delivery to the CNS by AAVs are currently underway and yielding promising results (Kaplitt et al., 2007; Muramatsu et al., 2010). The same properties that make AAVs promising for use in humans also make them useful for research into the structure and function of neural circuits (Betley and Sternson, 2011) and the neurotropic nature of AAVs has facilitated their use as anterograde fluorescent labels (Harvey et al., 2002; Leaver et al., 2006b; Leaver et al., 2006a; Foust et al., 2008; Planchamp et al., 2008; Blits et al., 2010; Vulchanova et al., 2010; Williams et al., 2012). This is consistent with the findings reported in this Chapter: the cells transduced by AAV-SpH appeared to be largely neuronal. In addition, the serotype used in this study, AAV2/5, has previously been reported to efficiently

transduce CNS tissue in a highly neuron-specific way (Burger et al., 2004; Paterna et al., 2004; Foust et al., 2008; Hutson et al., 2012). The CST is of particular interest to the field of spinal cord injury research due to its central role in voluntary motor function. Achieving CST transduction with viral vectors has been attempted with varying degrees of success (Yip et al., 2006; Foust et al., 2008; Blackmore et al., 2012; Hutson et al., 2012). In this Chapter, AAV-SpH injection into the cortex of the adult rat led to efficient CST transduction, as shown by a large area of transduction in the cortex and the presence of 2000-3000 transduced CST axons in the cervical spinal cord. In agreement with others (Harvey et al., 2002) we found that AAV was far superior to lentiviral brain injection, which led to a small area of transduction of SpH positive cells and no transduced axons were observed in the brainstem or spinal cord. It is likely that the relatively poor transduction observed after brain injection of LV-SpH can be attributed to the low titre achieved when vectors are made in-house. As we are not a specialist viral facility, our vectors are likely to be of lower titre and lower quality than those made by vector core facilities, for example, and significantly better cortical transduction may have been achieved using a commercially made lentiviral vector. Indeed, others have reported significantly better results using lentiviral vectors (e.g. Zhao et al., 2011), so it may not only be the type of vector used *per se* but also the quality of preparation that is most important.

4.4.2 *Potential uses of AAV-SpH to study the CST*

The delivery of fluorescent proteins by viral vectors for use as anterograde axonal labels is gaining in popularity and has recently begun to be employed as a tool for the study of axonal regeneration after injury (Andrews et al., 2009; Wu et al., 2009; Low et al., 2010; Wang et al., 2011). We have shown that following cortical injection of AAV-SpH, SpH can be visualised throughout the length of the corticospinal axon by

immunostaining for GFP. Thus, AAV-SpH can be used for the study of regeneration in the same way as traditional tracers, such as BDA. However, plastic changes following CNS injury are also known to be important and have been shown using electrophysiology (e.g. Bareyre et al., 2004; Massey et al., 2006; Cafferty et al., 2008). This Chapter has described the development of an anatomical tool for the quantification of synapses made by a specific neuronal tract, the CST. By accumulating in bright puncta at presynaptic sites, SpH allows investigation of the number and distribution of synapses. For example, the small ventral component of the CST was also labelled by AAV-SpH, which is useful as this tract has been reported to undergo extensive remodelling after dorsal CST injury (Weidner et al., 2001) and the nature of these changes may now be further explored.

A model that will lend itself to testing the usefulness of AAV-SpH is the pyramidotomy injury technique, which transects one half of the CST at the level of the pyramids in the brainstem, rostral to the spinal cord. The reactions of the uninjured tract can then be investigated as it projects in the spinal cord. This has been extensively used in the past to study the sprouting response in the spinal cord in response to treatments that promote growth or neutralise inhibitory factors in the spinal cord (e.g. Liu et al., 2010; Thallmair et al., 1998; Blochlinger et al., 2001). Using AAV-SpH the unilateral pyramidotomy model could be taken a step further, to investigate whether the regenerative sprouts are forming synapses.

As the results reported here are only for 3 brain-injected animals, further characterisation clearly remains to be carried out. However, once the technique is fully optimised it has the potential to go further than previous techniques examining functional connectivity, such as combining tract tracing with electron microscopy,

because of the potential use of the pHluorin to additionally examine the function of newly formed synapses, as well as their location.

4.4.3 *Using viral vectors to transduce sensory neurons*

Gene transfer to primary sensory neurons has been of interest to researchers interested in dissecting the mechanisms underlying nociceptive processes. In the present study, injection of two different AAVs into the sciatic nerve led to transduction of a small number of sensory neurons. AAV vectors expressing either SpH or a Cre-GFP fusion protein, when injected into the intact sciatic nerve, were found to transduce 1-3% of DRG neurons. It has recently been reported that transient demyelination of sciatic nerve axons improves retrograde transduction, by improving axonal access (Hollis et al., 2010). Similarly, we found that nerve crush could improve the number of GFP positive cells in the L5 DRG to 8%. This is lower than the 20% (Hollis et al., 2010) and 30% (Towne et al., 2009) transduction that have been achieved by others when injecting into the intact nerve. However, those studies used AAV2/1 and AAV2/6 vectors, which may be more effective at retrograde neuronal transduction than AAV2/5. Indeed, AAV2/5 has been shown to exhibit very poor retrograde transduction of motoneurons following injection into nerve or muscle, compared to AAV2/1 (Hollis et al., 2008). Very efficient transduction of DRG neurons using AAV2/5 has been achieved using direct DRG injection (Mason et al., 2010), consistent with its remarkable efficiency when directly injected into brain or spinal cord, as shown in this Chapter and elsewhere (Burger et al., 2004; Paterna et al., 2004; Blits et al., 2009). While 100% transduction of sensory neurons may be desirable for targeted gene delivery to act as a putative treatment, sparse labelling of afferents may be advantageous for the study of the structure of their central terminals.

4.4.4 *Potential uses of AAV-SpH to study sensory neuron projections*

A discussed above, AAV-SpH could be used to study plasticity of a major descending axonal tract after spinal cord injury. However, the versatile nature of viral vectors accommodates their application to a variety of neural tissues and experimental paradigms. One particular area where the application of the AAV-SpH virus may be useful is to elucidate the mechanisms underlying the development of neuropathic pain. One proposed mechanism is the sprouting of low threshold, large Aβ mechanoreceptors into superficial regions of the dorsal horn of the spinal cord, to synapse with nociceptive neurons (Woolf et al., 1992; Shortland and Woolf, 1993). However, this finding remains controversial as it has been claimed that the labelling, which was shown using CTB tracing, was artefactual (Hughes et al., 2003). Two recent studies have provided evidence for the role of synaptogenesis in the development of neuropathic pain by stereologically counting the number of synapses in the dorsal horn (Lin et al., 2011; Peng et al., 2010). This was achieved by immunostaining spinal cord sections for synaptophysin and counting the number of objects. This method did not identify the origin of the synapses. Using AAV-SpH, it would be possible to label primary afferents and map their terminations in the spinal cord. In addition, as the vector labels synapses, it becomes possible to measure synaptogenesis after neuronal injury.

4.4.5 *Technical considerations and future directions*

While viral vector delivery of SpH to neuronal populations is an exciting prospect for the study of synaptogenesis in a particular CNS tract or system, several considerations remain to be addressed. In this Chapter, the bright puncta that are observed on axons when SpH is expressed in neurons *in vitro* or *in vivo* have been referred to as synapses. However, whether these SpH-positive puncta are bona fide synapses remains unclear.

They may be vesicles transporting SpH along the axons and towards synapses, SpH aggregation in the axon as a side effect of overexpression or presynaptic specialisations with no corresponding postsynaptic site. To investigate the nature of the puncta, further work is required, including double immunostaining for pre- and postsynaptic markers as well as SpH. Colocalisation with a presynaptic marker (e.g. synapsin or synaptophysin) will rule out non-specific SpH accumulation. Close apposition with a postsynaptic marker (e.g. PSD-95) will confirm that the SpH-positive puncta are true synapses.

A recent study has used inducible, genetically encoded markers of the CST to study remodelling of the tract after SCI and specifically counted the numbers of boutons along CST collaterals, which were identified by their morphology and co-expression of synaptic markers, such as synapsin I (Lang et al., 2012). The morphology of the terminal CST branches and their boutons are very similar in appearance to those seen in the present study. The authors used the Thy1 promoter to drive YFP expression, meaning that YFP expression was Golgi-like, filling the cytoplasm and complicating the discrimination of boutons. As used in this Chapter, AAV-SpH accumulates much more brightly in the puncta than along the collateral branches, potentially making it a more useful tool to study synapses. However, it remains likely that the tool can be further improved. The SpH construct expressed by AAV-SpH is the same as that originally described by Miesenbock et al. (1998), but improved constructs have subsequently been described and may be considered in the future. For example, fusing the pHluorin reporter to a synaptic protein that is more localised to the synapse, such as synaptophysin or vGlut1-glutamate transporter, results in a significantly improved signal-to-noise ratio (Granseth et al., 2006; Balaji and Ryan, 2007; Dreosti and Lagnado, 2010).

This tool has been developed with a view to investigating the effect of nervous system injury on synapse location and function. However, it is possible injury could affect neuronal physiology and cause confounding issues. Axonal transport of SpH may be affected, such that if SpH is not visible in regenerating tips of axons, synaptogenesis may be underestimated. In addition, as with more traditional tracing techniques and in contrast with transgenic approaches, this approach is also subject to interanimal variability. Out of the 3 brain-injected animals the number of SpH-positive axons in the dorsal CST in the cervical spinal cord varied from ~1500 in the animal with the fewest positive axons to >5000 in the animal with the most. Viral vectors are additionally vulnerable to batch differences in titre, which may complicate their routine use. However, this variability can be controlled for by normalising to the number of transduced axons in the dorsal CST. Perhaps a more important issue is whether the number of presynaptic boutons corresponds to the number of axons in the tract. If there is great variability in this parameter, the ability to discriminate true injury or treatment effects from the natural interanimal variation in synapse number labelled will be compromised. Encouragingly, results gathered from brain-injected animals so far show that the number of synapses labelled is greater in those animals that have larger numbers of transduced CST axons and vice versa. In addition, the number of synapses labelled per spinal cord section is very consistent within an animal. However, the ratio of synapses to axons is not equivalent for the 3 animals. Further optimisation of the technique/quantification method will shed light on this issue.

A further problem that was encountered in these experiments is the low transduction efficiency of DRG neurons following sciatic nerve injection of AAV-SpH. A simple way to address this would be to crush the sciatic nerve at the time of AAV injection, as this was found to improve AAV-GFP-Cre transduction fourfold. However, introducing

a crush injury may not be desirable for pain studies as this additional injury alone may affect syanptogenesis and pain behaviour. Instead, constructing a vector using a different serotype may be the optimal solution. AAV2/1 and AAV2/6 are likely to be good candidates for alternative serotyping, according to previous studies (Hollis et al., 2008; Towne et al., 2009).

In this Chapter we describe the visualisation of synapse function *in vitro* and anatomical synapse distribution *in vivo*. The ultimate aim of the project is to test whether newly formed synapses are viable and capable of releasing neurotransmitter. If not, the regenerative sprouting described by many may be exactly what Cajal suspected – an abortive attempt at regeneration, with little functional meaning. Certainly, significant challenges lie ahead in the development of a paradigm for using SpH as a marker of synaptic activity. It is likely that an *ex vivo* slice preparation will be the starting point for further studies, which will allow manipulation of the preparation and flexibility regarding imaging and electrical stimulating methods.

4.4.6 *Conclusions*

Despite these reservations and the considerable challenges that lie ahead, AAV-SpH is a promising tool for the study of anatomical plasticity in the nervous system. The use of viral vector technology is an attractive alternative to injection of classic tracers or transgenic animals. AAVs in particular are easy to produce, produce little or no toxicity and transduce CNS tissue in an efficient, stable and neurotropic manner. Here we have shown that an AAV expressing a fluorescent synaptic protein can selectively label the CST and allow the visualisation and quantification of the number of synaptic terminals of its axons. AAV-SpH has the potential to become a powerful tool for investigating anatomical plasticity in the context of neurological disorders.

CHAPTER 5

General Discussion

5.1 Challenges to clinical translation

Traumatic SCI has catastrophic consequences, both for the individual living with lifelong disability and for society, in terms of the enormous healthcare and financial burden. Despite a wealth of research into regenerative medicine, no treatment that leads to any significant functional recovery has emerged. However, the scientific research effort continues to advance understanding of the neurobiological basis of SCI and has generated a plethora of prospective treatments, including pharmacological, biologic, surgical and rehabilitative interventions. A number of promising therapies have entered clinical trials, but most have been discontinued due to insufficient evidence of efficacy (Tator, 2006). An exception to this trend is the corticosteroid methylprednisolone, which is used routinely in some countries to minimise secondary damage and inflammation following SCI. However, the use of methylprednisolone has become increasingly controversial due to reports of limited clinical benefits and adverse side effect profile (Hurlbert and Hamilton, 2008). The complex and multifactorial nature of SCI makes it seem unlikely that targeting a single factor could lead to sufficient repair to generate functional recovery. However, significant plasticity occurs spontaneously after SCI, in parallel with spontaneous improvements in function (Raineteau and Schwab, 2001), so treatments that augment this endogenous process may represent a realistic therapeutic strategy. The extent of spontaneous improvement in humans with SCI is greater in those with a larger proportion of spared white matter, suggesting that it is reorganisation of this surviving tissue that mediates recovery (Blesch and Tuszynski, 2009). The patients with the greatest spontaneous recovery are those termed ASIA B, C or D by the American Spinal Injury Association (ASIA www.asia-spinalinjury.org). 60-80% of ASIA C patients undergo spontaneous improvement, compared with 20% of patients with clinically complete injuries (ASIA A patients). Evaluation of a putative

plasticity-promoting therapy remains challenging. The heterogeneity of human injury and the high rates of spontaneous improvement in function in patients with clinically incomplete injuries mean that it will be challenging to distinguish a treatment effect from endogenous changes, unless the drug effect is extremely striking (Fawcett et al., 2006; Blesch and Tuszynski, 2009). Any effect could be easier to detect in ASIA A patients, but since there is less white matter for the drug to act on, achieving an effect will be difficult. Clearly, significant challenges in the translation of plasticity-promoting treatments remain.

5.1.1 *ChABC as a putative treatment for SCI*

ChABC has been reproducibly shown, by independent laboratories, to have beneficial neuroanatomical and functional effects across different experimental models (Bradbury and Carter, 2011). However, many questions remain to be addressed in small animal models before further progress toward the clinic can be made. The majority of studies have employed an injury consisting of the transection of specific tracts, or transection of one half of the spinal cord, but very few studies have investigated efficacy in animal models more closely mimicking human injuries, the majority of which involve a spinal contusion. Of the few studies which have employed contusion or compression injuries, beneficial effects on locomotion and bladder function have been reported following treatment with ChABC, either given as a single therapy (Caggiano et al., 2005), or when applied as a combination therapy (Karimi-Abdolrezaee et al., 2010). The results reported in Chapter 3 of this Thesis support these findings. Clearly, significant differences in the neuroanatomy, behaviour and physiology exist between rodents and humans, limiting the predictive value of small animal models for human trials. ChABC has been delivered intrathecally to cats with hemisection injury and has yielded encouraging results that support the efficacy described in rodent models, with cats

exhibiting accelerated recovery (Tester and Howland, 2008). Nonhuman primates bear a far closer resemblance to humans and represent an essential stepping stone on the path to translation (Courtine et al., 2007). Studies assessing ChABC in nonhuman primate models of SCI have not yet been performed, but will provide valuable insight into the safety, efficacy and potential future use of ChABC in the clinic.

5.1.2 *Potential side effects of ChABC*

Studies in rodent models of SCI have so far failed to highlight any adverse effects of ChABC treatment, but it is important to consider possible deleterious effects of chronically elevated plasticity. For example, it is conceivable that unchecked sprouting could lead to the development of aberrant connections and an increased sensitivity to painful stimuli (Christensen and Hulsebosch, 1997). This has been specifically investigated, in this Thesis (Chapters 2 and 3) and elsewhere (Barritt et al., 2006; Galtrey et al., 2007; Karimi-Abdolrezaee et al., 2010), without any evidence for increased pain sensation. However, pain remains a potential complication to be aware of as translation progresses to larger mammals. Another adverse outcome that appears to be the result of neuroplasticity is autonomic dysreflexia, an exaggerated sympathetic reaction in response to sensory input below the lesion (Weaver et al., 2006). This condition can be dangerous and investigators should remain cautious when using treatments that promote plasticity (Brown and Weaver, 2012). So-called maladaptive plasticity has also been implicated in spasticity, cardiac arrhythmias and bladder, bowel and sexual dysfunction (Collins et al., 2006; de Groat and Yoshimura, 2006; Johnson, 2006; Nout et al., 2006). Thus, although neuroplasticity represents a promising avenue for the promotion of functional recovery after SCI, it is important to note that this process may also underlie maladaptive changes.

5.1.3 *Timing of intervention*

Anatomical data from Chapter 3 support a neuroprotective function for ChABC by showing reduction in the characteristic lesion pathology that develops following contusion injury and also showed evidence of anatomical plasticity below the injury. If neuroprotection is the main mechanism underlying improved function then this effect is likely to be reduced if there is a delay in ChABC expression. Although previous studies have reported efficacy with delayed ChABC delivery (Carter et al., 2011; Wang et al., 2011), these studies employed injury models that have less traumatic pathology than the contusion injury. It is vital that experimental treatments in animal models of SCI are applied with an appropriate delay and that the therapeutic window for the effectiveness of treatments is established. So far, the majority of studies have investigated ChABC applied immediately after injury, but it would be helpful, and perhaps more realistic, to also investigate the effect of subacute application (72 hours to 14 days after injury, Tator, 2006). In addition, this will help elucidate the magnitude of the neuroprotective versus the plasticity-promoting effect of ChABC.

5.1.4 *Viral delivery of ChABC – advantages and limitations*

The prospect of long-term CSPG digestion using LV-ChABC makes this a promising tool for future studies, overcoming the considerable difficulties of delivering an active enzyme repeatedly to the spinal cord. However, translation of lentiviral vectors into the clinic is associated with significant biosafety concerns. Lentiviral vectors integrate randomly into the host cell genome, leading to the danger of insertional mutagenesis and the formation of cancers, which have been seen in animal models and in human clinical trials (Li et al., 2002; Hacein-Bey-Abina et al., 2003; Themis et al., 2005). These concerns may be circumvented by the use of integration-deficient vectors (Yanez-Munoz et al., 2006) or the use of regulatable viral vector, where gene

expression can be switched off (Gossen and Bujard, 1992; Gossen et al., 1995). An advantage of gene delivery via viral vector includes long-term expression of the transgene. In the case of lentiviral vectors, expression has been reported for periods of up to 16 months (Kordower et al., 2000; Bienemann et al., 2003; Balaggan et al., 2006). This is useful in the context of disease treatment as the therapeutic protein can act over an extended period of time, especially in the case of an enzyme, such as ChABC where digestive activity rapidly decreases within 1-2 days after a single injection (Lin et al., 2008). Chapter 3 describes the use of three different lentiviral vectors expressing ChABC and demonstrates that there is considerable spatiotemporal variability in CSPG digestion between vectors. Furthermore, the extent of CSPG digestion appeared to correlate with the degree of improvement in axonal conduction after contusion injury. In Chapter 3 of this Thesis, LV-ChABC enabled CSPG digestion throughout the spinal cord, including in lumbar segments where the sciatic nerve spinal circuitry is located and which may have contributed to the development of A-fibre wind-up. The effectiveness of gene transfer in the nervous system could be compromised by an immune response to the vector particles (Wong et al., 2006). However, lentiviral vector injection has been associated with minimal, asymptomatic inflammation (Kordower et al., 2000; Mazarakis et al., 2001) and systemic immunity is not induced after their application to the CNS (Abordo-Adesida et al., 2005). Thus developments in enhancing the safety features of lentiviral vectors, together with a lack of immunogenicity, make lentiviral vectors potential attractive candidates for gene therapy applications and several clinical trials are underway (Snyder et al., 2010).

5.1.5 *Potential alternatives to ChABC*

Although it is well established that CS-GAG chains are responsible for CSPG-mediated inhibition, the mechanisms that govern the inhibition of axonal growth are not resolved.

It is becoming increasingly clear that CS-GAGs with different sulphation motifs are differentially up-regulated after injury, suggesting distinct roles for the various polysaccharides (Gilbert et al., 2005; Properzi et al., 2005; Lin et al., 2011). A recent study by Brown et al. (2012) identified a sugar epitope, CS-E, that is primarily responsible for the inhibitory effects of CSPGs and activates growth inhibitory signalling pathways via its interaction with a known CSPG receptor, PTPσ (Brown et al., 2012). Furthermore, the authors show that the use of a CS-E blocking antibody can enhance axonal regeneration *in vivo*, presenting the opportunity to block the inhibitory effects of CSPGs by targeting a specific CS epitope, rather than by removing all CS-GAGs. These findings also present the opportunity to specifically target the enzyme that synthesises CS-E, sulfotransferase Chst15, by developing a small molecule antagonist. Another enzyme that has been suggested as a target to prevent the synthesis of CSPGs is xylosyltransferase, which is responsible for the glycosylation of CSPGs. Targeting this enzyme has been shown to lead to increased axonal growth in a model of SCI in vivo (Grimpe and Silver, 2004; Hurtado et al., 2008). Overall, using antibodies or small molecules to target specific CSPG components may offer an alternative approach to treatment which may be less invasive and immunogenic, as well as having fewer potential side-effects than LV-ChABC.

5.2 Conclusion

The results of this Thesis are in support of other preclinical studies which suggest that ChABC is a promising treatment for SCI. Moreover, this work has demonstrated that, by using a gene delivery approach to optimise delivery and achieve stable degradation of CSPGs, we can promote significant functional repair in a clinically relevant

traumatic model of SCI, and can promote extensive plasticity of reflexes when applied to the spinal cord after injury to the peripheral nerve or the spinal cord. Although significant challenges remain before clinical translation of ChABC as a therapeutic strategy, nevertheless these data show promise that progress is being made towards achieving that goal.

Chapter 6

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